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Weidong Le *Editor*

Autophagy: Biology and Diseases

Clinical Science

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Preface

Although only 4 years have passed since the second edition of *Autophagy—Biology and Diseases* was published in 2015, basic and clinical studies of autophagy have developed rapidly in China. In recent years, new academic achievements have been made, and many new techniques and methods have been established successfully. Since July 2015, a total of 22,401 papers on autophagy have been published and indexed by PubMed, of which 8860 were contributed by Chinese researchers. Facing the challenges of new science, new theory, and new technologies, in order to keep up with the frontier and accurately grasp the development direction of autophagy research, we have further revised and enriched the content of *Autophagy—Biology and Diseases*.

Considering the continuity of the editing and taking into account the innovative thinking of young scientists as well as newly developed technologies in the research field of autophagy, Profs. Zheng-Hong Qin, Wei-Dong Le, and Zhi-Ping Xie are invited as co-chief-editors of the third edition of this book. We have the privilege to invite more than 30 well-known domestic experts, including academician Jia Fan of the Chinese Academy of Sciences and academician Xiang-Mei Chen of the Chinese Academy of Engineering, to participate in compilation. These scholars or clinicians are experienced in autophagy studies and are the mainstay of the academic team of autophagy research in China. At the same time, the editing and preparation of this book has been guided by Prof. Daniel Klionsky, Academician Hong-Yang Wang and Prof. Hong Zhang, three authoritative scholars in autophagy research. All these contributors have their own research directions and specialties. Their academic thoughts will be presented in different chapters and add luster to the contents of this book.

Compared with the previous two editions, the third edition of *Autophagy—Biology and Diseases* has a considerable expansion in the contents. We try to introduce the basic theory and clinical knowledge of autophagy more comprehensively and systematically, and to reflect the most recent progress and achievements in autophagy research. Especially, the third edition will pay more attention to the introduction of autophagy research techniques and methods, which will be more practical and instructive for guiding the beginners to master autophagy research techniques and

tools faster. We hope that the third edition of this book will enable readers to appreciate the real situation and broad research prospects of autophagy, and provide useful tools and references for promoting basic and clinical research of autophagy in China.

At the same time, in order to increase the international impacts of Chinese autophagy research, we also commissioned Springer Nature Publishing House to publish the English version of this book. Although this book covers the basic theory, methods, clinical significance, and frontier findings of autophagy research, it is still difficult to cover all aspects of autophagy research. Limited to the level of knowledge and literary accomplishment, there are inevitably omissions and deficiencies in the content and editing work of this book. All criticisms and corrections will be greatly appreciated and your comments and criticisms will help us perfect the reprinted version.

July 2019

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Abbreviations

3'UTR	Three prime untranslated region
3-MA	3-Methyladenine
4E-BP	Eukaryotic translation initiation factor 4E (eIF4E)-Binding Protein
4-HPR	N-4-Hydroxyphenyl retinoid
5-FU	5-Fluorouracil
5-TH	5-Hydroxytryptamine
α1-AT	Alpha-1-Antitrypsin
α-syn	α-synuclein
A2M	α2-Macroglobulin
AAT	1-Antitrypsin
ABCA1	ATP Binding cassette transporter A1
ABT	Androgen blockade therapy
ACTH	Adrenocorticotropic Hormone
AD	Alzheimer's disease
ADHD	Attention deficit hyperactivity disorder
ADNP	Activity-dependent neuroprotective protein
ADPKD	Autosomal dominant polycystic kidney disease
AEC II	Alveolar epithelial cell II
AFP	α-Fetal-associated protein
AG	Autoimmune gastritis
AGEs	Advanced glycation end products
AgRP	Anabolic agouti-related peptide
AICAR	5-Aminoimidazole-4-carboxamide ribonucleotide
AICD	APP intracellular domain
AIEC	Adherent-invasive Escherichia coli
AIF	Apoptosis-inducing factor
AKI	Acute kidney injury
AKT	Protein kinase B (PKB)
ALDH1	Acetaldehyde Dehydrogenase 1

ALL	Acute lymphoblastic leukemia
ALS	Amyotrophic lateral sclerosis
Ambra1	Activating molecule in beclin1-regulated autophagy1 (autophagy and beclin 1 regulator 1)
AML	Acute myeloid leukaemia
AMPK	Adenosine 5′-monophosphate (AMP)-activated Protein Kinase
Ang	Angiotensin
ANGII	Angiotensin II
ANXA7	Annexin A7
APL	Acute Promyelocytic Leukemia
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
ARHI	Aplasia Ras homolog member I
AS IV	Astragaloside IV
AS	Atherosclerosis
ASC	Apoptotic speck protein containing a caspase recruitment domain
ASD	Autism Spectrum Disorder
ASICs	Acid-sensing ion channels
AT1R	Angiotensin type 1 receptor
ATD	α-1-Antitrypsin deficiency
ATF4	Activating transcriptional factor 4
ATF6	Transcription activator 6
Atg	Autophagy-related gene
Atg101	Autophagy-related protein 101
ATG12	Autophagy-related gene 12
Atg13	Autophagy-related protein 13
ATG16L	Autophagy-related 16-like 1
ATG5	Autophagy-related gene 5
ATG7	Autophagy-related gene 7
Atg7	Autophagy-related protein 7
Atg8	Autophagy-related protein 8
ATGL	Adipose triglyceridelipase/desnutrin
ATO	Arsenic trioxide
ATP	Adenosine triphosphate
ATRA	All-trans retinoic acid
ATZ	Alpha 1-antitrypsin Z
Aβ	Amyloid–β protein
BA 10	Brodmann Area 10
BA 22	Brodmann Area 22
BACE	β-site APP cleaving enzyme
BAEC	Bovine aortic endothelial cells
Baf A1	Bafilomycin A1

Bak	Bcl-2 homologous antagonist/killer
Bax	BCL2-associated X protein
BCAAs	Branched-chain amino acids
Bcl-2	B-cell lymphoma-2
Bcl-xl	B-cell lymphoma-extra large
BCSCs	Breast cancer stem cell
BDNF	Brain-derived neurotrophic factor
BH3	Bcl-2 homology 3
Bif-1	Bax-interacting Factor 1 (or Endophilin B1)
BIN1	Bridging integrator
BITC	Benzyl isothiocyanate
BMDM	Bone marrow derived macrophages
BNIP3	BCL2 interacting protein 3
BPAN	Beta-helical protein-related neurodegenerative diseases
BRAF	Proto-oncogene B-Raf
BRC	Bromocriptine
BSE	Bovine spongiform encephalopathy
CA8	Carbonic anhydrase-related protein VIII
CA16	Coxsackievirus A16
CAB	Cabergoline
CAF	Cancer-associated fibroblasts
CAG	Chronic atrophic gastritis
CaMKII	Calcium/calmodulin-dependent protein kinase II
CaMKIV	Calcium/calmodulin-dependent protein kinase IV
CARD	Caspase-activating and recruitment domain
CASP1	Caspase1
Caspase	Cysteine aspartic acid-specific protease
Caspase-7	Cysteine-aspartic protease-7
Cav-1	Caveolin-1
CB2R	Cannabinoid receptor 2
CB3	Coxsackievirus B3
CB4	Coxsackievirus B4
CBS	Cystathionine-β-synthase
CBZ	Carbamazepine
CCI-779	Temsirolimus
CD	Crohn's disease
CDCP1	CUB domain-containing protein 1
CDK	Cyclin-dependent kinase
CEP	Cepharanthine
CERS1	Ceramide synthase 1
CEs	Cholesteryl esters
CF	Cystic pulmonary fibrosis
CGI58/ABHD5	αβ-Hydrolase domain-containing protein 5
CHOP	C/EBP-homologous Protein
CIC	Cancer initiation cell

CJD	Creutzfeldt–Jakob disease
CKD	Chronic kidney disease
CLDN1	Claudin 1
CLL	Chronic lymphocytic leukemia
CLP	Cecal ligation and puncture
CLU	Clusterin
CMA	Chaperone-mediated autophagy
CML	Chronic myelogenous leukaemia
CMV	Cytomegalovirus
CNAG	Chronic non-atrophic gastritis
CNP	C-type natriuretic peptide
CNS	Central nervous system
CoCSC	Colon cancer stem cell
COPD	Chronic obstructive pulmonary disease
Cox	Cyclooxygenase
CPEB	Cytoplasmic polyadenylation element binding
CPEO	Chronic progressive external ophthalmoplegia
CQ	Chloroquine
CR1	Complement receptor 1
CREB	cAMP response element-binding protein
CryAB	αB-Crystallin
CS	Cigarette smoke
CS	Cordyceps Sobolifera
CSC	Cancer stem cell
CSE	Cigarette smoke extract
CTCs	Circulating tumour cells
CTS	Cathepsin
CVB3	Coxsackievirus B3
CVD	Cardiovascular diseases
CWD	Chronic wasting disease
CysC	CystatinC
DA	Dopamine Agonist
DAC	Dacomitinib
DAG	Diacylglycerol
DAMPs	Damage-associated molecular patterns
DAPK1	Death-associated protein kinase 1
DARPP-32	Dopamine- and cAMP-regulated neuronal phosphoprotein of 32 kD
DCN	Decorin
DCs	Dendritic cells
DEDD	Death effector domain-containing DNA-binding protein
DEN	Diethylnitrosamine
DEPTOR	DEP domain-containing mTOR-interacting protein
DFNA5	Deafness, Autosomal Dominant Nonsyndromic Sensorineural 5

DHA	Dihydroartemisinin
DHEA	Dehydroepiandrosterone
DISC	Death-inducing signaling complex
DISC1	Disruption of disrupted-in-Schizophrenia 1
DKD	Diabetic kidney disease
DLB	Dementia with Lewy bodies
DMP	3,5-Dimethylpyrazole
DNM2	Dynamin 2
dNTP	deoxynucleotide triphosphates
DOX	Doxorubicin
DRAM	DNA damage-regulated autophagy modulator
Dram1	DNA damage-regulated autophagy modulator protein 1
DRD2	Dopamine Receptor D2
DRD5	Dopamine Receptor D5
DRPLA	Dentatorubral-pallidoluysian atrophy
DSS	Dextran sulphate sodium
DTCs	Disseminated tumour cells
DTNBP1	Dystrobrevin-binding Protein 1
ECM	Extracellular matrix
ECT	Electroconvulsive Therapy
EDD	Endothelium-dependent dilatation,
eEF2K	Eukaryotic elongation factor 2 kinase (Eukaryotic factor 2 kinase)
EGCG	Epigallocatechin-3-gallate
EGFR	Epidermal growth factor receptor
EGFR-TKIs	Epidermal growth factor receptor tyrosine kinase inhibitor
Egr-1	Early growth response-1
EMT	Epithelial to mesenchymal transition
EndMT	Endothelial-to-mesenchymal transition
Env	HIV Envelope glycoproteins
EPC	Endothelial progenitor cell
ER	Endoplasmic reticulum
ER	Estrogen receptor
ERAD	ER-associated protein degradation
ERK	Extracellular-regulated Protein Kinases
ERS	Endoplasmic reticulum stress
ESRD	End-stage renal disease
Etk	Epithelial and endothelial tyrosine kinase
EV71	Enterovirus 71
FAK	Focal adhesion kinase
FDA	Food and Drug Administration
FEZ1	Fasciculation and Elongation Protein 1
FFA	Free fatty acid
FFI	Fatal familial insomnia
FGF10	Fibroblast growth factor 10

FIP200	Focal adhesion kinase family interacting protein of 200
FKBP12	FK506 binding protein 1A
FMF	Familial Mediterranean fever
Fmr1	Fragile X mental retardation 1
FMRP	Fragile X mental retardation protein
FN	Fibronectin
FOXO1	Forkhead box O1
FQ	Ferroquine
FSGS	Focal segmental glomerulosclerosis
FSH	Follicle-stimulating hormone
FTD	Frontotemporal dementia
FTLD	Frontotemporal lobar degeneration
GAAC	General amino acid control
GABA	γ-aminobutyric acid
GABARAP	Gamma-aminobutyric acid type A receptor-associated protein
Gb3	Globotriaosylceramide
GBM	Glioblastoma
GBPs	Guanine nucleotide-binding regulatory protein
GCA	Grancalcin
GCX	Glycocalyx
GEF-H1	Guanine nucleotide-exchange factor
GFR	Glomerular filtration rate
GH	Growth hormone
GlcNAc	N-acetylglucosamine 1-phosphotransferase
GOLD	Global initiative for chronic Obstructive Lung Disease
GPI	Gycosylphosphatidylinositol
GRA	Glucocorticoid receptor antagonist
GRP78	Glucose-regulated Protein 78
GSC	Glioma stem-like cells
GSDMD	Gasdermin D
GSK	Glycogen synthase kinase
GSK-3β	Glycogen synthase kinase-3β
GSS	Gerstmann–Straussler–Scheinker
GWAS	Genome-wide association study
GβL	G protein β-subunit-like protein
H_2O_2	Hydrogen peroxide
HAP 1	Htt-associated protein 1
HBP1	HMG-box transcription factor 1
HBsAg	Hepatitis B s antigen
HBV	Hepatitis B virus
HCC	Hepatocarcinoma
HCMV	Human cytomegalovirus
HCQ	Hydroxychloroquine
HCV	Hepatitis C virus
HD	Huntington's disease

HDAC	Histone deacetylase
HDAC6	Histone deacetylase 6
HDACIs	Histone deacetylase inhibitors
HDL	High-density lipoprotein
HEK	Human embryonic kidney
HF	Heart failure
HFS	High-frequency stimulation
HIF-1	Hypoxia-inducible factor-1
HIF-1α	Hypoxia-inducible factor 1-α
HIV	Human immunodeficiency virus
HK-2	Human kidney proximal tubular cell line
HMGB1	High-mobility group box protein 1
HMG-CoA-R	3-Hydroxy-3-methyl glutaryl-coenzyme A reductase
HO-1	Heme oxygenase-1
HP	Helicobacter pylori
HPCs	Hepatic progenitor cells
HPO	Hypothalamus–pituitary–ovary
HRCP	Human retinal capillary pericytes
HRE	Hypoxia response element
HSCs	Hepatic stellate cells
HSL	Hormone-sensitive lipase
Hsp27	Heat shock protein 27
HSP70	Heat shock protein 70
HSV-1	Herpes simplex virus 1
Htt	Huntingtin
HUVECs	Human umbilical vein endothelial cells
IBD	Inflammatory bowel disease
IC	Intermediate colitis
ICC	Interstitial cells of Cajal
ICC-DMP	Deep muscular ICC
ICC-IM	Intramuscular ICC
ICC-MY	Myenteric ICC
ICC-SM	Submucosal ICC
ICH	Intracerebral hemorrhage
ICP34.5	Infected cell protein 34.5
IDDM	Insulin-Dependent diabetes mellitus
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IGF1	Insulin-like growth factor1
IKK	Inhibitor of nuclear factor kappa-B kinase
IKKβ	Inhibitor κ B kinase β
IL	Interleukin
IL-1β	Interleukin 1β
IL-2	Interleukin-2
IL-6	Interleukin-6

IM	Imatinib
iNOS	Inducible nitric oxide synthase
IPF	Idiopathic pulmonary fibrosis
IR	Ischemia reperfusion
IRE1	Inositol-requiring enzyme 1
IRF	Interferon response factor
IRG	Immunity-related GTPase family
IRGM	GTPase family M protein
IRI	Ischemia/reperfusion injury
IRS1	Insulin receptor substrate 1
ITB	Intestinal tuberculosis
ITGB4	Integrinβ4
JNK	c-Jun N-terminal kinase
KCs	Kupfer cells
KEAP1	Kelch-like ECH-associated protein 1
KSS	Kearns–Sayre syndrome
LAMP1	Lysosomal-associated membrane protein 1
LAMP2	Lysosome-associated membrane protein 2
LAMP2A	Lysosome-associated membrane protein type 2A
LARP1	La ribonucleoprotein domain family member 1
LC3	Microtubule-associated protein light chain 3
LDL	Low-density lipoprotein
LDs	Lipid droplets
LH	Luteotropic Hormone
LHON	Leber's hereditary optic neuropathy
LOX-1	Oxidized low-density lipoprotein receptor-1
LPS	Lipopolysaccharide
LRP	Low-density lipoprotein receptor
LSD	Lysosomal Storage Disorders
LTD	Long-term depression
LTP	Long-term potentiation
MAP1B	Microtubule-associated protein 1B
MAP1 LC3	Microtubule-associated Protein 1 Light Chain 3
MAP1S	Microtubule-associated proteins 1S
MAPK	Mitogen-activated protein kinase
MAVS	Mitochondrial antiviral-signaling protein
MCA	3-Methylcholanthrene
MCT4	Monocarboxylate transporter 4
MDCK	Madin-Darby canine kidney epithelial cells
MDD	Major Depressive Disorder
MDR1	Multidrug resistance 1
MDS	Myelodysplastic syndrome
MEFs	Mouse embryonic fibroblasts
MELAS	Mitochondrial myopathy, encephalomyopathy, lactic acidosis, stroke-like symptoms

MERRF	Myoclonic Epilepsy with Ragged Red Fibers
MF	Mifepristone
MFN2	Mitofusin 2
MGL	Monoacylglycerol lipase
MHC	Major histocompatibility complex
mHtt	Mutant Htt protein
MJD	Machado–Joseph disease
MKP-1	Mitogen-activated protein kinase phosphatase 1
mLst8	Mammalian Lethal with SEC13 Protein8
MMP	Matrix metalloproteinase
MMP2	Matrix metalloproteinase 2
MNGIE	Myoneurogenic gastrointestinal encephalopathy
MOMP	Mitochondrial outer membrane permeabilization
MPs	Microparticles
MPS	Mucopolysaccharidoses
MPT	Mitochondrial permeability transition
MPTP	Mitochondrial permeability transition pore
MSC	Mesenchymal stem cell
Mst1	Mammalian Ste20-like kinase 1
MTA1	Metastasis-associated protein 1
MTB	Mycobacterium tuberculosis
mtDNA	Mitochondrial DNA
MTOC	Microtubule organizing centre
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian Target Of Rapamycin Complex 1
MTs	Microtubules
MTX	Methotrexate
mVps34	Yeast Vacuolar Protein Sorting Defective 34
NAC	N-acetyl cysteine
NAD	Nicotinamide adenosine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
Naf-1	Neutrient-deprivation autophagy factor-1
NAFLD	Nonalcoholic fatty liver disease
NAMPT	Nicotinamide phosphoribosyltransferase
NAP	An 8-Amino-acid Peptide from ADNP
NARP	Neuropathy, ataxia, retinitis pigmentosa, and ptosis
NASH	Nonalcoholic steatohepatitis
NAT8L	N-acetyltransferase 8-like protein
NCLs	Neuronal Ceroid-Lipofuscinoses
NCX	Na–Ca exchange
NDEL 1	Nuclear Distribution Gene E Homologue-like 1
NDP52	Nuclear dot protein 52
NF1	Neurofibromatosis type 1
NFTs	Neurofibrillary Tangles
NHE1	Sodium–hydrogen antiporter 1

NLRP3	NOD-like receptor protein 3 (NOD-like receptor family, pyrin domain containing-3)
NLRs	Nucleotide-binding oligomerization domain (NOD)-like receptor
NMDA	N-methyl-D-aspartate receptor
NPC	Niemann-Pick type C disease
NPCs	Neural precursor cells
NRAMP1	Natural resistance-associated macrophage protein 1
Nrf2	Nuclear factor (erythroid-derived 2)-related factor 2
NRGI	Neuregulin-1 (NRG1) Genes
NSAIDs	Non-steroidal Anti-inflammatory Drugs
NSCLC	Non-small cell lung cancer
NSF	N-ethylmaleimide sensitive factor
NSP4	Non-structural protein 4
OGD	Oxygen-glucose deprivation
OIS	Oncogene-induced senescence
OPCA	Olivo Ponto Cerebellar Atrophy
OPTN	Optineurin
oxLDL	OXIDIZED low-density lipoprotein
p38 MAPK	p38 Mitogen-activated Protein Kinase
P62	Recombinant Sequestosome 1(SQSTM1)
PA	Pituitary adenoma
PAD	Peripheral arterial disease
Pae	Paeoniflorin
PAMP	Pathogen-associated molecular patterns
PARP	Poly (ADP-ribose) polymerase
PC	Phosphorylcholine
PCD	Programmed cell death
PCOS	Polycystic Ovarian Syndrome
PC-PLC	Phosphatidylcholine-specific phospholipase C
PCSK9	Proprotein convertase subtilisin/kexin type 9
PD	Parkinson's disease
PDAC	Pancreatic ductal adenocarcinoma
PDCD5	Programmed cell death 5
PDE4A4	Phosphodiesterase 4A4
PDGF	Platelet-derived growth factor
PE	Phosphatidylethanolamine
PEBP1	Phosphatidylethanolamine binding protein 1
PERK	Protein kinase R-like endoplasmic reticulum kinase
PF	Paeoniflorin
PFS	Progression-free survival
PG	Prostaglandin
PGC1	Peroxisome proliferator-activated receptor-γ coactivator-1α
pH	Potenz hydrogenion (Hydrogen Ion Concentration)
PI3K	Phosphatidylinositol 3-kinase

PI3KIII	Phosphatidylinositol 3-kinase
PI3KR4	Phosphoinositide-3-Kinase Regulatory Subunit 4
PICALM	Phosphatidylinositol binding clathrin assembly protein
PINK1	PTEN-induced kinase 1(PTEN induced putative kinase 1)
PKC	Protein kinase C
PKD	Polycystic kidney disease
PKM2	M2 pyruvate kinase isoform
PLD1	Phospholipase D1
PLG	Piperlongumine
PLIN 2	Perilipin 2
PolyQ	Polyglutamine
POMC	Catabolic proopiomelanocortin
PP2A	Protein phosphatase 2A
PPAR	Peroxisome proliferators-activated receptors
PPAR-γ	Peroxisome proliferator-activated receptor-γ
PRL	Prolactin
PrP^{c}	Cellular isoform of prion protein
PrP^{Sc}	Scrapie isoform of prion protein
PRR	Pattern recognition receptor
PRR	Pro-renin receptor
PSA	Puromycin-sensitive aminopeptidase
PSEN	Presenilin
p-Ser317-ULK1	Phospho-ULK1 (Ser317) Antibody
p-Ser93-Beclin1	Phosphorylate Beclin 1 at Ser93 Antibody
p-Thr172-AMPKα	Phospho-AMPK Alpha-1 (Thr172) Polyclonal Antibody
PTCs	Proximal tubular epithelial cells
PTEN	Phosphatase and tensin homolog deleted on Chromosome Ten
PTK2	Protein tyrosine kinase 2
PUMA	p53 upregulated modulator of apoptosis
PV	Poliovirus
QSOX1	Quiescin Sulfhydryl oxidase 1
RAB7	Ras-related protein Rab-7a
Raptor	Regulatory-associated protein of mTOR
RB1CC1	RB1 inducible coiled-coil 1
RCC	Renal cell carcinoma
RCT	Reverse cholesterol transport
RGCs	Radial glial cells
RhoA	Ras homolog family member A
Rictor	Rapamycin-insensitive companion of mTOR
RIP1	Receptor-interacting protein kinase 1
RIP3	Receptor-interacting protein kinase 3
ROCK	Rho kinase
RORα	RAR-related orphan receptor A
ROS	Reactive oxygen species

RTK	Receptor Tyrosine Kinase
SAA	Serum amyloid A
SAH	Subarachnoid hemorrhage
SBMA	Spinobulbar muscular atrophy
SCA	Spinocerebellar atrophy
SCA1	Spinocerebellar atrophy1
SCA3	Spinocerebellar atrophy3
SCA7	Spinocerebellar atrophy7
SCLC	Small cell lung cancer
SCRAB	Scavenger receptor B
SCVs	Salmonella-containing vacuoles
SCZ	Schizophrenia
Ser	Serine
SFN	Sulforaphane
SFTRC	Surfactant protein-C
Shank3	SH3 and Multiple Ankyrin Repeat Domains Protein
SIN1	SAPK Interacting Protein1
SIRT1	Silent mating type information regulation 2 homolog 1 (silent information regulator 1, NAD-dependent deacetylase sirtuin-1)
SNARE	Soluble NSF attachment protein receptor
SNP	Single-nucleotide polymorphisms
SOD1	Superoxide dismutase1
SP	Senile Plaque
SPARC	Secreted protein acidic and rich in cysteine
spv	Salmonella plasmid virulence
SQSTM1	Sequestosome 1
SR	Serine Racemase
SREBP-2	Sterol regulatory element binding protein-2
SRL	Somatostatin Analogue
STAT3	Signal transducer and activator of transcription 3
STING	Stimulator of interferon genes
STOP	Stable Tubule Only Polypeptide
STZ	Streptozocin
SUMO	Small ubiquitin related modifier
T2DM	Type 2 diabetes
T3SS	Type III secretion systems
TACE	Transarterial chemoembolization
TAM	Tumour-associated macrophage
TBK1	TANK-binding kinase 1
TCA	Tricarboxylic acid
TES	Transmissible spongiform encephalopathies
TFEB	Transcription factor EB
TG	Triglyceride
TG	Tripterygium glycoside

TG2	Transglutaminase 2
TGF-β	Transforming growth factor-β
TGF-β2	Transforming growth factor-beta 2
TIA1	T-cell-restricted intracellular antigen 1
TICE	Transaminase cholesterol excretion
TK	Tyrosine kinase
TKI	Tyrosine kinase inhibitors
TLR	Toll-like receptor
TLR4	Toll-like receptor 4
TMBIM6	Transmembrane Bax inhibitor motif containing 6
TMZ	Temozolomide
TNF-α	Tumor necrosis factor-α
TORCH	Toxoplasma Others Rubella Cytomegalo Herpes
Tp53	Tumor protein p53
TPT	Topotecan
TRAF6	TNF receptor-associated factor 6
TRAIL	TNF-related apoptosis-inducing ligand
TRAV6	T cell receptor alpha variable 6
TRIF	Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta
TSC	Tuberous sclerosis complex
TSC2	Tuberous sclerosis complex 2
TSH	Thyroid-secreting Hormone
TUNEL	TdT-mediated dUTP Nick-End Labeling
Ub	Ubiquitin
UC	Ulcerative colitis
UCP1	Uncoupling protein 1
UIMs	Ubiquitin interacting motifs
ULK	Unc-51 like kinase
ULK1	Unc-51 like autophagy activating kinase 1 (UNC-51 like kinase 1)
UNC-76	Caenorhabditis Elegans Protein UNC-76
UPR	Unfolded protein response
UUO	Unilateral ureteral obstruction
UVRAG	UV irradiation resistance-associated Gene
Vac8	Vacuolar protein 8
VacA	Vacuolating cytotoxin A
VCP	Valosin containing protein
VD	Vascular dementia
VDAC	Voltage-dependent anion channel
VDAC1	Voltage-dependent anion channel 1
VEGF	Vascular endothelial growth factor
VEGFA	Vascular endothelial growth factor A
VIP	Vasoactive intestinal peptide
VPSPr	Variably protease-sensitive prionopathy

VSMC	Vascular smooth muscle cell
Wdfy3	WD repeat and FYVE domain containing 3
WDR45	(or WIPI4) WD Repeat Domain 45
WPB	Weibel-Palade body
WT	Wide type

Part I
Autophagy and Neuropsychological Disorders

Autophagy is a crucial lysosomal degradation and recycling process in the eukaryotic cell, responsible for maintaining cellular function and homeostasis of cell survival and cell death. This dual role played by autophagy raises the question of whether this process is protective or destructive? Deregulated autophagy at different steps of this cellular process, whether over-activated or depressed, has been proposed to be associated with various neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease. Recent observations of impaired autophagy also appeared in psychiatric disorders such as schizophrenia and bipolar disorder suggesting an additional contribution to the pathophysiology of mental illness. Here we review the current understanding of autophagy's role in various neuropsychiatric disorders and, hitherto, the prevailing potential autophagy-related therapeutic strategies for their treatment.

Chapter 1
Autophagy and Alzheimer's Disease

Sheng Chen, Qinming Zhou, You Ni, and Weidong Le

Abstract Alzheimer's disease (AD) is the most common type of dementia and is characterized by progressive cognitive decline. Increasing evidence has demonstrated that the autophagic process plays an important role in AD. In this chapter, we will discuss the role of autophagy in the pathogenesis of AD and other types of dementia, including dementia with Lewy bodies (DLB), frontotemporal dementia (FTD), vascular dementia (VD) and prion diseases. In addition, we will discuss autophagy-targeted therapies as future treatments for AD.

Keywords Alzheimer's disease · Dementia · Autophagy

Alzheimer's disease (AD) is a very common type of dementia that is characterized by progressive cognitive decline. According to the World Health Organization, more than 40% of people older than 85 years ultimately develop AD. The typical manifestation of AD is progressive memory loss. In the early stage of the disease, patients may experience difficulties performing activities of daily living. The core neuropathologic features of AD include neurofibrillary tangle formation and neuritic amyloid plaque deposition. AD is difficult to treat. Conventional treatments fail to slow disease progression, although cholinesterase inhibitors, including tacrine, donepezil and galantamine, can be used to treat symptoms. Ideal treatment strategies for AD should not only focus on improving symptoms but also target the mechanisms of AD to protect neurons from degeneration by either reducing the burden of senile plaques or preventing neurofibrillary tangle formation. Autophagy is an intracellular lysosomal degradation process that plays an important role in cell growth and development. Autophagy has multiple physiological functions, including protein degradation, organelle turnover and responses to stress. Increasing evidence has suggested that autophagic dysfunction may play an important role in the pathogenesis

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of AD. In this chapter, we will discuss the role of autophagy in the pathogenesis of AD and other types of dementia, including dementia with Lewy bodies (DLB), frontotemporal dementia (FTD), vascular dementia (VD) and prion diseases. In addition, we will discuss autophagy-targeted therapies for the treatment of AD.

1.1 The Role of Autophagy in the Pathogenesis of AD

Lysosomes are single-membraned vesicular organelles that originate from the Golgi apparatus. They contain 60 acidic enzymes that can hydrolyze polysaccharose, phospholipids, nucleic acids and proteins. The pH of the enzymatic reactions that occur in lysosomes is 5. During aging and disease, it is difficult for proteins to be digested by posttranslational enzymes and through chemical modifications, and they remain in the residual bodies. In lysosomes, cathepsins, which have nearly 24 different catalytic levels for different peptide bonds, can also rapidly degrade most proteins into amino acids over a wide range of acidic pH values (Wolfe et al. 2013). Substrates to be digested enter the lysosome by phagocytosis and autophagy. Phagocytosis is the process by which extracellular invaders, such as bacteria and food, can be decomposed into small fragments to be reused or excreted. Autophagy is a cellular process for the turnover of cytoplasmic constituents, including organelles, proteins and pathogens. Both phagocytosis and autophagy are related to APP processing and the pathogenesis of AD.

Autophagy is a lysosomal degradative process by which damaged organelles and macromolecules are degraded. It is a biological phenomenon for maintaining basic cellular functions. It can be induced in response to a variety of stressors, such as nutrient starvation, oxidative stress and infection. The degradation products of autophagy, such as amino acids, nucleotides and free fatty acids, can be recycled for the synthesis of new molecules and energy. Autophagy can remove misfolded proteins, harmful metabolites, aging mitochondria and so on, and it has an anti-aging effect. Hence, autophagy is an important pathway for maintaining cellular homeostasis. In contrast to other cells, neurons, as postmitotic cells, cannot dilute toxic substances through mitosis. Some autophagy-related proteins (Beclin 1, Atg7 and Atg12) participate in the late onset of many neurodegenerative diseases. Because the expression of autophagy-related proteins decreases as age increases, autophagy is usually active in young neurons. Autophagy is divided into three types: microautophagy, macroautophagy and chaperone-mediated autophagy (CMA). Hereafter, macroautophagy, which is the greatest contributor of the three to the pathogenesis of AD, will be referred to by the general term autophagy. Many proteins related to AD are metabolized by autophagy.

Although abnormal protein accumulation in the brains of AD patients suggests that autophagy, a metabolic pathway involving protein circulation and transportation, may play a role in the pathogenesis of AD, not enough attention has been paid to it due to the limitations of experimental technologies. Once researchers observed

autophagosomes and autophagic vesicles in the brain neurons of AD patients and the accumulation of autophagic vesicles of different stages in malnourished neurites, autophagy became a hot topic in the field of AD pathobiology.

1.1.1 Aβ and Autophagy

An increasing number of studies have indicated that Aβ plays a vital role in the incidence and development of AD. The neurotoxicity of Aβ involves many complex mechanisms, such as destroying Ca^{2+} homeostasis, inducing the production of free radicals, damaging the function of K^+ channels and worsening the inflammatory response. First, we will introduce the process of Aβ generation. APP is a transmembrane glycoprotein widely distributed in the cellular membrane of many tissues, and it exists as a transmembrane receptor. It includes a long extracellular N-terminal segment and a short intracellular C-terminal segment. Although the biological function of APP is not well understood, it is important for molecular signal transduction and cell adhesion. There are two pathways by which APP is hydrolyzed. In the first pathway, APP is spliced into a soluble N-terminal domain (sAPPα) by α-secretase and then it is secreted out of the cell. The intracellular C-terminal fragment (αCTF) is further spliced into small soluble peptides and an APP intracellular domain (AICD) by γ-secretase. Previous studies have found that the AICD regulates gene transcription. In the second pathway, APP is hydrolyzed into $Aβ_{40}$ and $Aβ_{42}$. APP is secreted out of the cell after being spliced into a soluble APP N-terminal domain (sAppβ) by β-site APP cleaving enzyme (BACE). The intracellular C-terminal fragment (βCTF) is spliced into a large amount of $Aβ_{40}$ and a small amount of $Aβ_{42}$ by γ-secretase. γ-Secretase, which is composed of presenilin, nicastrin, APH-1 and PEN-2, exists in many parts of the cell, including the cell membrane, early and late endosomes, autophagosomes and lysosomes. The generation of intracellular Aβ is the main source of diffuse Aβ and deposited Aβ fibrils. The amount of soluble Aβ in endosomes and lysosomes greatly increases before the extracellular deposition of Aβ. The Aβ level is increased in AD mice, but mice develop cognitive impairment before extracellular plaque formation. Aβ is neurotoxic before being released from the cell. After being secreted, extracellular soluble Aβ and accumulated Aβ cause pathological effects by binding with surface receptors, which can affect the bilayer structure of the cell membrane directly or affect the function of lysosomes after cellular uptake. In addition, in the very early stage of AD, autophagosomes can product toxic Aβ, which accumulates in neurons. Immunofluorescence experiments have revealed that APP, Aβ and PS1 are present in the autophagosomes of AD mice. The production of Aβ increases after autophagic induction, while Aβ aggregation decreases upon autophagic inhibition.

Studies on the autophagic origin of AD have provided some evidence of the role of the endosome–lysosome system in AD. The endosome–lysosome pathway plays an important role in APP processing and Aβ synthesis (Sarah et al. 2010). Cells can monitor the environment, take up extracellular nutrients and regulate the expression

of surface receptors through endocytosis. After endocytosed molecules are sorted, some return to the cell membrane, some are transported to the Golgi body for further processing and some are transported to late endosomes and lysosomes for degradation. The first cellular pathological process that occurs in the brains of AD patients is the activation of the endocytosis pathway. Neurons in sensitive brain regions exhibit progressive endocytosis abnormalities in the form of increasing early endosome volume. Abnormalities in the lysosomal pathway occur earlier than the pathological manifestation of neurofibrillary tangles and senile plaques. The function of the lysosomal system is upregulated in vulnerable cells. The number of lysosomes increases as the expression of lysosome hydrolases increases. The highly expressed hydrolases also include cathepsins, which participate in Aβ generation directly and indirectly. As AD development progresses, lysosomal dysfunction leads to the formation of vacuoles and Aβ aggregation. When the degeneration of vulnerable neurons occurs, intracellular Aβ is released into the extracellular space in vacuoles. Intracellular Aβ release is the main source of extracellular Aβ precipitation. Lysosomal abnormalities can be caused by the induction and dysfunction of the endocytosis pathway, but they can also be affected by the autophagy pathway. There is a definite overlap between endocytosis and the autophagy pathway in neurons. Late-stage endosomes usually merge with autophagosomes. The deep understanding of autophagy has attracted our attention to studying the effect of autophagy in the pathogenesis of AD.

The autophagy–lysosome system is an important pathway for Aβ degradation under physiological conditions. However, some researchers have found that Aβ can be generated by the autophagy–lysosome system under pathological conditions or during aging (Li and Le 2010). A large number of studies have illustrated how Aβ is produced by endosomes, the Golgi body and the endoplasmic reticulum. Increasing attention has been paid to another pathway (the autophagy–lysosome system) in the generation of Aβ. It is clear that autophagy is important for APP processing and Aβ generation. Researchers have found that a large amount of Aβ is present in the autophagosomes of abnormal neurons. Autophagosomes contain Aβ and are immunopositive for its intermediate precursor, βCTF, and they exhibit high PS-dependent γ-secretase activity. Autophagy activation and the enhanced splicing of APP by γ-secretase also occurs in the brains of hypoxia-treated APP/PS1 transgenic mice. The increase in the number of autophagosomes provides more space for Aβ production. Aβ develops into oligomers and fibrils easily in the acidic environment of lysosome-related organelles. At the same time, it has been indicated that autophagic activation can improve Aβ generation in in vitro cell cultures. Autophagosomes from different tissues contain numerous components of γ-secretase and strong γ-secretase activity. Autophagy is significantly induced when mouse fibroblasts are treated with rapamycin or starvation. A large number of γ-secretase complexes are transported from endosomes and the endoplasmic reticulum to autophagic vacuoles. At the same time, autophagic vacuoles are the largest compartments with strong γ-secretase activity. Cells produce a large amount of Aβ, specifically twice as much as that produced by autophagy-inhibited cells. Serum starvation can also induce the autophagy of neurons well and increase Aβ generation more than three times.

Le Weidong et al. reported that autophagy activation and enhanced APP splicing by γ-secretase occurs in the brains of hypoxia-treated APP/PS1 transgenic mice (Li et al. 2009). Transmembrane protein PS1, as one of the important components of the γ-secretase complex, plays a vital role in Aβ generation. Mutations in *PS1* are one of the most common mutations that cause familial AD. Autophagy dysfunction can increase the expression of PS1 and the activity of γ-secretase. The close relationship between *PS1* and autophagy has drawn great interest from many researchers. It has been found that *PS1* can interact with the intracellular adhesion factor telencephalin (TLN). In *PS1* knockout cells, it is difficult for autophagy to clear TLN, which has a long half-life, and this may be attributed to the failure of autophagic vacuoles and lysosomes to fuse. *PS1* is necessary for autophagy. Dysfunctions in lysosomal acidification and cathepsin activation lead to deficits in autophagosome clearance in the neurons of *PS1* knockout mice. The V0a1 subunit of V-ATPase plays a key role in lysosomal acidification, and its transport from the endoplasmic reticulum to the lysosomal membrane requires the involvement of *PS1*. Mutant *PS1* loses the ability to bind with the unglycosylated V0a1 subunit so that it cannot be transported to the lysosome. Then, the proteins to be cleared by autophagosomes cannot be acidulated and digested by lysosomes, ultimately leading to autophagosome accumulation (Lee et al. 2010). Lysosomal acidification and the accumulation of autophagosomes is also exhibited in the fibroblasts of familial AD patients carrying *PS1* mutations. Some researchers obtained embryonic fibroblasts from *PS1* knockout mice. A significant increase in autophagy and an increase in the number of autophagosomes and lysosomes occur in embryonic fibroblasts. However, there is an obvious decrease in autophagy-mediated protein degradation. In addition, protein degradation is slightly changed after treatment with a γ-secretase complex inhibitor in normal cells. This indicates that dysfunctions in the *PS1*-mediated autophagy–lysosome pathway are independent of the γ-secretase complex.

Aβ that is produced by autophagosomes is mainly transported to lysosomes for degradation by cathepsin. Some researchers have found that the expression of Aβ increases significantly in the brains of cathepsin B knockout mice and that it decreases significantly in the brains of mice in which cathepsin B is upregulated. It is interesting that Aβ generated by autophagosomes depends more on lysosomal degradation in human neurons than in rodents. Some Aβ generated by autophagosomes is released from the cell by the exocytosis of multivesicular bodies. In the normal brain, autophagy plays a small role in the maintenance of basal Aβ content related to effective autophagic clearance and lysosomal degradation to inhibit Aβ accumulation. After autophagy is induced rapidly in fibroblasts, autophagosomes quickly aggregate, and the function of lysosomes is delayed. The delay in the function of autophagosomes provides an opportunity for a large amount of Aβ to be produced and released from the cell. Neurons have a powerful ability to clear autophagic vesicles, which can be quickly and effectively cleared even after autophagy is severely induced. Nevertheless, when the maturation process of autophagic vesicles is impaired or delayed, autophagic vesicles also aggregate in large numbers and produce a large amount of intracellular and extracellular Aβ. Autophagy is involved in the extracellular secretion of Aβ. The quantitative analysis of extracellular Aβ protein has revealed that Aβ

protein secretion is reduced by 90% in autophagy-deficient mice. After autophagy is restored, Aβ protein secretion returns to normal. In addition, in Atg7 knockout mice, the extracellular secretion of the Aβ protein is greatly reduced, which leads to the aggregation of the Aβ protein in cells. Studies have reported that familial AD patients with *PS1* and *PS2* mutations have more severe lysosomal system disorders than those of sporadic AD patients, and their levels of cathepsins D and B are also higher. In CRND8 transgenic AD mice with autophagic lysosomal fusion disorder, researchers knocked out cystatin B, an endogenous inhibitor of lysosomal proteases. They found that autophagolysosomal pathway function was restored and that the deposition of Aβ and the levels of other proteins degraded by autophagolysosomes were significantly reduced. Because lysosomal function was restored in the CRND8 transgenic AD mice, the levels of $A\beta_{40}$, $A\beta_{42}$ and extracellular senile plaques in their brains were significantly reduced. Their study not only demonstrated the important role of autophagy–lysosome pathway disorders in the pathogenesis of AD but also provides evidence for the possibility of treating AD by restoring lysosomal function.

The induction of autophagy in the early stage of AD can transfer a large number of APP-rich metabolic substrates to the autophagy metabolic pathway, and it can speed up the circulation of proteins and organelles in damaged or regenerated synapses. At the same time, the activation of autophagy reflects an increase in the demand of nutrients, such as proteins, by neurites, and it reflects a self-protective response against apoptosis. In the late stage of AD, excessive APP and Aβ can lead to the dysregulation of autophagy. The toxic effect of Aβ interferes with the anchoring effect between the endoplasmic reticulum and microtubules, and it reduces the stability of the microtubules. In addition, there are a large number of autophagosomes and the abnormal aggregation of various immature autophagic vacuoles in the cortical and hippocampal neurites of late-stage AD mice and patients, indicating that autophagosome fusion to lysosomes is inhibited in the late stage of AD (Nixon 2007). PS1 is involved in the transport and synthesis of APP, which can be converted into Aβ. PSEN1 mutations lead to the increased production of Aβ polypeptides and the formation of Aβ plaques. At the same time, PS1 also participates in the fusion of autophagosomes and lysosomes, while mutations in PS1 lead to autophagosome-lysosomal formation disorders. During normal growth and the regeneration of neurites, immature autophagic vacuoles are retrogradely transported through the cell to fuse to lysosomes. Then, the contents of the autophagic vacuoles are rapidly degraded after fusion. The process from autophagic vesicle formation to lysosome fusion and digestion is so rapid and efficient that no obvious autophagic vesicle formation can be observed. In the late stage of AD, autophagosomes and other autophagic vacuolar subtypes are clearly observed in malnourished neurites. Their formation and existence demonstrate abnormalities in autophagic vesicle transport and maturation. If lysosomes are blocked from degrading proteins and organelles through autophagy in circulating cells, the damage to neurons will be further aggravated. For example, mitochondrial degradation disorder leads to the accumulation of lipofuscin. It has also been found that, under conditions of enhanced autophagy, the expression of lipofuscin is upregulated in many components of the lysosomal system, and this upregulation continues until the late stage of neuronal degeneration.

The severe accumulation of autophagosomes, other autophagic vacuolar subtypes and hydrolase-positive dense bodies occurs in swollen dystrophic processes. They become the main organelles in neurites (Nixon and Yang 2011). Neurites containing many autophagic vacuoles are extensively neuroinflammatory and dystrophic and characteristically swollen. However, these neurites are not typical in other neurodegenerative diseases that do not produce Aβ. These processes provide a good research direction for a more in-depth study of AD pathogenesis.

In summary, autophagy plays multiple roles in AD. It can not only produce proteins such as Aβ but also promote the release of Aβ. We should further explore whether changes in autophagy are a cause or a consequence of AD.

1.1.2 Tau Metabolism and Autophagy

Another major pathological feature of AD is the formation of large neurofibrillary tangles (NFTs) in nerve cells. Recent studies have indicated that the formation of NFTs is primarily associated with the hyperphosphorylation of tau. Tau is a microtubule-associated protein involved in the assembly and stabilization of microtubules. Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), as well as cyclin-dependent kinase 5 (Cdk5) and glycogen synthase kinase 3β (GSK-3β), plays important role in the abnormal hyperphosphorylation of tau protein. Hyperphosphorylated tau proteins assemble into double-stranded filaments that are 820 nm wide and have a perimeter of 80 nm, and they are highly insoluble and unable to bind to microtubules due to conformational changes. This assembly causes the failure of tubulin polymerization, resulting in a final collapse of the microtubule network (Sahara et al. 2008). The formation and deposition of Aβ can induce mitochondrial toxicity, which causes Ca^{2+} overload and subsequent CaMKII activation. CaMKII activation further leads to the hyperphosphorylation of tau and the inhibition of tau-induced tubulin assembly, thus resulting in neuronal dysfunction or, even worse, apoptosis.

Despite the fact that the ubiquitin–proteasome system is a main pathway for tau degradation, recent studies have revealed autophagy as an alternative pathway for the degradation of tau. The same conclusion was drawn from another study on vacuolar myopathy. Chloroquine, as a weak base, can be taken up by muscle cells and be concentrated mainly in acidic organelles, such as lysosomes. The long-term administration of chloroquine can cause lysosomes to swell and induce vacuolar myopathy. Interestingly, the accumulation of many AD-related molecules, such as tau, Aβ, APP and ApoE, has been found in the vacuoles of affected muscle fibers, indicating a high similarity between the pathogenesis of myopathy and AD, especially in the process of protein deposition caused by the dysfunction of lysosomes. The normal tau protein can be degraded by lysosomes, but the degree of phosphorylation of the tau protein directly affects its binding to receptors on the lysosomal membrane. Hyperphosphorylated tau cannot be efficiently digested by lysosomes, and it forms fibrillary tangles that are toxic to neurons. Autophagy may also affect the phosphorylation status of the

tau protein. The tau protein is hyperphosphorylated in autophagy-deficient mice, and the restoration of autophagy in these mice reduces hyperphosphorylated tau. Some studies have found that rapamycin can clearly eliminate mutant tau protein (Uddin et al. 2019). Rapamycin, an inhibitor of mTOR (mammalian target of rapamycin), activates the autophagy pathway by inhibiting the activity of mTOR. However, it is not well established whether rapamycin abolishes the accumulation of the tau protein by activating autophagy, although it is known that rapamycin can reduce the aggregation of abnormally folded proteins by inhibiting protein synthesis in autophagy-deficient fibroblasts. After autophagy is inhibited by 3-methylindole (3-MA), the activity of proteasomes and calpain significantly improves, and the level of the tau protein decreases. Therefore, researchers speculate that the autophagy–lysosome pathway may not be the primary pathway for tau protein metabolism.

As early as 1967, Suzuki discovered the abnormal accumulation of tau and subcellular structures in damaged axons from brain tissues from AD patients. However, it was not until 2005 that Nixon et al. identified these abnormally aggregated structures as autophagic vesicles by immunocolloidal gold and electron microscopy techniques. Scientists have discovered lysosomes with abnormal function and activity in the brains of transgenic mice with mutant human tau by electron microscopy and enzymatic activity detection. More interestingly, abnormalities in lysosomal distribution have also been observed in neuronal cell lines stably transfected with mutant human tau. The wild-type tau protein can compete with kinesin for binding sites on microtubules, and cells control kinesin-dependent cell transport along microtubules by regulating the level of tau. Normally, lysosomes are mainly distributed in the central region of the cell. Since the mutated tau protein easily aggregates and has poor binding affinity for microtubules, the inhibition of the tau protein greatly attenuates kinesin-dependent forward transport. When cells overexpress pathologically mutated tau, lysosomal transport becomes so active that lysosomes are dispersed throughout the cell. The transport of lysosomes, as well as many processes in the autophagy pathway, including the transport of autophagosomes, depends on the forward movement of microtubules. The excessive phosphorylation of tau can cause the instability and abnormal assembly of cytoskeletal proteins, resulting in the abnormal transport and maturation of autophagosomes. This leads to the abnormal aggregation of autophagosomes within axons and a series of pathological effects. When the expression of the tau protein is decreased rapidly in a cellular model of Niemann-Pick disease type C, it can reduce the induction of autophagy and the flow of autophagic vesicles. It has also been demonstrated that tau can regulate the normal function of the autophagy–lysosome pathway by stabilizing the function of microtubules. Because of its high level of phosphorylation and abnormal folding, the tau protein impairs the normal aggregation of tubulin and microtubule-mediated transport function and further affects the fusion of autophagosomes and lysosomes. Protein phosphatase 2A (PP2A) is an important phosphatase that regulates the phosphorylation of the tau protein. Its dysfunction can directly lead to the hyperphosphorylation of the tau protein. Other studies have also found that okadaic acid can significantly increase the formation of autophagosomes in rat neurons. Additionally, okadaic acid, as an

inhibitor of PP2A, can promote the phosphorylation of the tau protein, which indirectly proves that the accumulation of autophagosomes and abnormal autophagic function may be the result of tau dysfunction.

1.1.3 Other Factors Associated with AD and Autophagy

A large number of studies have found that abnormalities in autophagic lysosomal function can affect not only the production of Aβ in neurotoxicity but also the degradation cycle of other molecules involved in the pathogenesis of AD, further increasing the severity of AD.

There are a large number of γ-secretase components PS1 in the autophagic vacuoles of AD patients and mice. PS1 is not only related to the overproduction of Aβ but also to pathological changes in the autophagy–lysosome system. Fibroblasts from AD patients with *PS1* mutations exhibit a large accumulation of abnormal autophagic vacuoles after autophagy activation, but the degradation cycle of long-lived proteins in cells is weakened rather than enhanced due to activation of autophagy. Autophagy-mediated protein degradation is found to be significantly reduced in embryonic cells obtained from *PS1* and *PS2* knockout mice, but this reduction can be reversed by the reintroduction of the *PS1* gene. These studies indicate that *PS1* plays a pivotal role in the normal function of autophagy. In AD patients carrying the mutant *PS1* gene, autophagic function is not maintained, leading to the dysregulation of autophagic vacuole maturation, an extensive accumulation of autophagic vacuoles, and the formation of inclusions that cannot be degraded and recycled effectively (Zareshahabadi et al. 2015).

BECN1 is an autophagy-related gene that encodes the Beclin 1 protein. Its mRNA level in the brain tissues of AD patients is reduced, especially in areas with severe pathological changes (such as the hippocampus). The expression of Beclin 1 declines progressively as AD progresses (Pickford et al. 2008). The expression of the proapoptotic protein caspase-3, which can cleave the Beclin 1 protein, increases in the brains of AD patients, leading to deficits in autophagosome formation. When AD mice overexpressing human APP are crossed with mice with impaired Beclin 1 expression, the offspring exhibit autophagic dysfunction and disease aggravation. In addition, the expression of Beclin 1 may also affect nonneuronal cells in AD. In the microglia of AD patients, the expression of Beclin 1 is decreased, and the number of vesicle transport complexes is decreased. The abnormal expression of Beclin 1 may affect the localization of vesicle transport complexes on phagosomes and receptor-mediated phagocytosis.

BACE is the β-secretase for APP, and it cleaves APP to produce βCTF, a direct precursor of Aβ. Inhibitors of lysosomal proteases can cause the accumulation of endogenous ectopic BACE, and the inhibition of lysosomal hydrolase activity also causes the redistribution and accumulation of BACE in lysosome-associated membrane protein 2 (LAMP2) positive cellular vesicles. In the late stage of AD, lysosome function is impaired due to the toxic effects of a large amount of intracellular Aβ.

The degradation of BACE in damaged neurons is gradually reduced, resulting in an increase in the β-cleavage of APP and more βCTF production.

ApoE ε4 acts as a risk factor for AD and can promote intracellular Aβ production. The expression of Aβ42 in the neurons of APP transgenic mice lacking the ApoE gene is significantly reduced (Nixon 2013). The colocalization of the ApoE ε4 allele and cathepsin D has been observed in human brain neurons and astrocytes cultured in vitro. In addition, there is high-intensity ApoE ε4 staining in senile plaques; the ApoE ε4 allele is colocalized with cathepsin D in brain slices from patients with AD. These findings suggest that cathepsin D may be an important enzyme for the degradation of ApoE ε4 in the brains of patients with AD. Once the autophagy–lysosomal pathway undergoes progressive functional decline, the degradation of ApoE ε4 is hindered, and the amount of Aβ produced in neurons is thus further altered.

There are increased levels of mitochondrial markers (lipoic acid) and cytochrome oxidase 1 in the cytoplasm of the cortical neurons of patients with AD, and most of these markers are localized to autophagic vesicle-like structures. When the function of autolysosomes is impaired, the degradation of mitochondria is insufficient, and lipofuscin continues to accumulate, further exacerbating the dysfunction of damaged neurons.

1.2 Autophagy-Targeted Therapy for the Treatment of AD

The most important question is whether autophagy-targeted therapy can postpone the progression of the disease. Increasing evidence has shown that autophagy modulation can effectively ameliorate neurodegeneration. Thus, various reagents targeting autophagy have been investigated. A large number of studies have confirmed that AD is actually a protein disease with abnormal folding and the aggregation of proteins. Abnormal autophagy plays an important role in the pathogenesis of AD. Therefore, adjusting and controlling autophagic lysosome function may be a promising treatment strategy for AD. In vitro, nicotine has been found to inhibit cell death caused by Aβ by enhancing autophagy. In vivo, Beclin 1, a key molecule in early autophagy in AD, is significantly downregulated. In Beclin 1 gene knockout mice, lysosomes are greatly damaged, and this damage is associated with significant neurodegenerative changes. Reduced Beclin 1 levels in the *APP* transgenic AD mice may result in the increased deposition of extracellular senile plaques and neurodegeneration. These findings indicate that autophagy defects can seriously affect APP metabolism. Autophagy has a protective effect against AD. However, other studies have found that the level of autophagy and the production of Aβ increase significantly after exposure to hypoxia and other environmental stimuli. This suggests that the activation of autophagy, especially in the late stage of AD, leads to more aggregation of neuronal autophagic vesicles and the worsening of the disease.

Although the induction of autophagy can enhance the clearance of aggregated proteins and protect cells and animal models against the toxic effects of these mutant

proteins, maintaining the active state of autophagy at an appropriate level rather than overregulating autophagy is a treatment target for these protein diseases. The serine/threonine protein kinase (mTOR) protein is a major negative regulator of the autophagic pathway. In AD mice, inhibiting the expression of the mTOR protein reduces the deposition of the Aβ protein in the brain and improves memory. In contrast, the overactivation of the mTOR protein increases the aggregation of the Aβ protein. Therefore, mTOR is generally considered an important therapeutic treatment target for autophagy. mTOR is a core component made of two distinct complexes, mTOR complex 1 (mTORC1) and mTORC2. The role of mTORC1 is to negatively regulate autophagy. However, mTORC2 has the opposite function. Under normal conditions, autophagy is suppressed by mTOR. mTORC1 phosphorylates and inhibits the core autophagy complex composed of ULK1, Atg13 and FIP200. Rapamycin, as an inhibitor of mTOR, can indeed accelerate the clearance of intracellular aggregated proteins, but it must be applied with caution because the rapid circulation of organelles in this process is a high cost for cells. The effect of rapamycin application is different in different stages of disease. In the early stage of AD, plaques and neurofibrillary tangles have not yet formed. The long-term application of rapamycin can activate autophagy, significantly reduce the formation of plaques and neurofibrillary tangles in the brains of mice, and improve learning and memory in mice (Majumder et al. 2011). In the late stage of disease, plaques and neurofibrillary tangles have already formed. Even when rapamycin is used to activate autophagy long-term, the therapeutic effect is very limited, neuropathological changes in mice cannot be alleviated, and learning and memory in mice cannot be improved (Uddin et al. 2019). Rapamycin interacts with immunophilin FK506-binding protein (FKBP12) to form a complex that inhibits the kinase activity of mTORC1, thus inducing autophagy. The rapamycin analogue temsirolimus also shows similar effects in AD mice. Temsirolimus is an FDA-approved compound for the treatment of renal cell carcinoma. It can promote the clearance of Aβ protein through an mTOR-dependent pathway in HEK293-APP695 cells and APP/PS1 transgenic mice. Temsirolimus can promote the clearance of hyperphosphorylated tau protein in an SH-SY5Y cell model and in brain tissues from P301S transgenic mice. In addition, in behavioral tests conducted in APP/PS1 and P301S mice, temsirolimus improved spatial learning and memory (Jiang et al. 2014). Latrepirdine is also an autophagy-regulating drug that targets mTOR. It has been shown to significantly improve cognitive impairment in patients with AD in small-sample clinical trials, but it failed to achieve success in phase III clinical trials. Because mTOR is a very important cellular protein that participates in many important cellular functions, the long-term use of mTOR inhibitors to treat diseases may have adverse effects, limiting its application in some patients. Furthermore, some drugs that indirectly target mTOR, such as nilotinib, can stimulate the AMPK pathway in an mTOR-dependent manner to induce autophagy. Nilotinib is a tyrosine kinase inhibitor that can enhance the interaction between parkin and Beclin 1 and thus promote the clearance of Aβ. Arctigenin is a natural product of *Arctium lappa*. It can inhibit Aβ production and enhance the clearance of Aβ by inhibiting the AKT/mTOR pathway and thus activating the autophagy process.

In neurodegenerative diseases, some drugs can activate autophagy through an mTOR-independent pathway. Lithium, a drug used to treat bipolar disorder, has been shown to activate autophagy by inhibiting inositol monophosphatase and reducing inositol and phosphoinositol levels. Lithium can significantly reduce neurodegeneration in a Drosophila model of HD and significantly delay the course of ALS in ALS mice and ALS patients. Scyllitol is also an autophagy inducer that targets the inositol signaling pathway. Clinical trials have shown that it has potential therapeutic effects in AD. In CRND8 transgenic mice, it can not only inhibit the aggregation of the Aβ protein but also reduce the aggregation of autophagic vesicles. Similar drugs, such as L-690, carbamazepine and sodium valproate, can also inhibit the aggregation of the Aβ protein and reduce its toxicity in cellular and mouse models. However, there has not been a clinical trial to confirm these therapeutic effects. GTM-1 is a novel small molecule that can attenuate Aβ oligomer-induced neurotoxicity by enhancing the autophagic process in an mTOR-independent manner. Trehalose is a natural disaccharide. It has been demonstrated to reduce the deposition of Aβ by enhancing autophagy. In addition, trehalose has also been shown to have the potential to prevent the progression of ALS in animal models of ALS by activating autophagy. Trehalose can improve learning impairment by reducing Aβ deposition in APP/PS1 mice. Since trehalose is free of toxic effects even at high concentrations, this agent may become a promising drug for future clinical applications.

In addition, several studies have shown that an increase in the intracellular calcium concentration can inhibit autophagy, while a decrease in the intracellular calcium concentration can promote autophagy by affecting the formation of autophagosomes and autophagy–lysosome fusion. Some FDA-approved calcium channel antagonists (such as verapamil and nimodipine) can induce autophagy. Recently, some drugs have been tested in AD patients and models of AD, but the efficacy is still uncertain (Li et al. 2017). In cellular and animal models of AD, for example, isradipine can increase autophagy by inhibiting calcium influx and the expression of the α-1C subunit of L-type voltage-dependent calcium channels and alleviate the burden of intracellular Aβ aggregation.

The efficacy of gene therapy using lentiviruses or adenoviruses for the treatment of some neurodegenerative diseases has been tested. In recent years, an Aβ adenovirus oral vaccine, which can improve the clearance of the Aβ protein in brain tissues and restore cognitive function by increasing autophagy levels in animal models of AD, was developed. Therefore, regulating autophagy by viral delivery may be an important treatment approach for AD. Beclin 1, an important protein encoded by the BECN1 gene, is involved in the initiation of autophagy. Its expression level in the brain tissues of patients with AD is significantly decreased. Some studies injected lentiviruses encoding Beclin 1 into 6-month-old APP transgenic mice for 8 weeks. The results showed that the expression level of Beclin 1 increased, while the intracellular immune response to the Aβ protein decreased significantly (Kou and Chen 2017).

In autophagy-targets treatments for AD, the two sides of the autophagy pathway should be fully considered based on the perspective of autophagic flux. The activation of autophagy may be beneficial or harmful at different stages of disease and may

even damage the stability of the intracellular environment and lead to autophagic cell death. In summary, more in-depth basic and clinical trials are needed to develop strategies for the treatment of AD based on autophagic targeting.

1.3 Autophagy in Other Types of Dementia

Dementia is not a specific disease. It is a term that describes a group of symptoms associated with a decline in memory or other thinking skills severe enough to reduce a person's ability to perform everyday activities. AD accounts for 60–80% of dementia cases. AD is the most common cause of progressive dementia, followed by dementia with Lewy bodies (DLB), frontotemporal dementia (FTD) and vascular dementia (VD). The pathophysiological mechanisms of these types of dementia are also related to the inadequate removal of abnormally aggregated proteins in the brains of patients. For example, in the brains of patients with DLB, in addition to the presence of the pathological changes that occur in AD, such as senile plaques and neurofibrillary tangles, there are also a large number of Lewy bodies composed of α-synuclein (α-syn). The pathological mechanism of FTD is the massive accumulation of phosphorylated tau protein or the TDP-43 protein in the frontotemporal lobe of patients. As mentioned above, autophagy is an important pathway for the clearance and degradation of abnormally aggregated proteins in cells. Changes in the autophagy level and protein abnormalities associated with the autophagy–lysosome pathway are significantly related to the occurrence of various types of dementia (Kragh et al. 2012).

TDP-43 is a conserved and widely expressed nuclear protein. Its main physiological function is to participate in the selective splicing of gene exons. In the brains of patients with FTD, PD and DLB, the TDP-43 protein is transported from the nucleus to the cytoplasm and forms TDP-43-positive inclusion bodies. A large number of TDP-43 C-terminal fragments with a molecular weight of 25 KD have been found in lesioned brain regions, and the excessive expression of these C-terminal fragments in cultured neuronal cell lines is sufficient to induce the translocation and aggregation of the endogenous full-length TDP-43 protein in the cytoplasm of vesicles. When the autophagy is inhibited in cells, the aggregation of the C-terminal fragments is increased; when the autophagy is activated in cells, the aggregation of the C-terminal fragments is significantly reduced, and the translocation of TDP-43 from the nucleus to the cytoplasm is greatly reversed. This evidence suggests that TDP-43 C-terminal fragments play a role in the pathogenesis of these neurodegenerative diseases and are closely related to autophagy.

1.3.1 DLB and Autophagy

DLB mainly manifests as fluctuating cognitive impairment, parkinsonism and psychiatric symptoms, including visual hallucinations. The pathological feature of DLB is the formation of diffuse Lewy bodies in the cerebral cortex and subcortical nuclei. Lewy bodies are mainly composed of abnormally aggregated α-syn. They are spherical, eosinophilic bodies in the cytoplasm of neurons. The progressive aggregation of α-syn in the central nervous system plays a very important role in the development of DLB. α-Syn is not only clustered in the substantia nigra but also abundant in the limbic area, frontal cortex and subcortical nuclei. Recent studies have reported that the aggregation of α-syn into Lewy bodies can cause synaptic damage and neurodegeneration, and the removal of aggregated α-syn may play a key role in the treatment of DLB. The autophagy–lysosome pathway is the main process by which α-syn aggregates are removed. Other studies have indicated that α-syn accumulation can interfere with the normal functions of the autophagy pathway and eventually cause nerve degeneration. Mutant α-syn has been documented to block autophagic pathways. The overexpression of α-syn has been found to be associated with autophagic disorders and neurodegeneration in cell culture and transgenic mice. Studies have suggested that abnormalities in specific targets of autophagy pathways can cause DLB. Elevated levels of mTOR and decreased levels of Atg7 have been found in the brains of patients with DLB and in the brains of transgenic mice with α-syn mutations. Rapamycin or the overexpression of Atg7 can alleviate the aggregation of α-syn and its related symptoms. In addition, recent studies have shown that blocking mTOR by overexpressing Thor (4E-BP) can alleviate the neuropathological manifestations of PD in models. Rapamycin can also activate 4E-BP in vivo to alleviate the pathological manifestations of PD resulting from PINK1 or PARKIN gene mutations. In addition, animal studies have shown that the knockout of Atg7 can cause neurodegeneration. However, it is not clear how the elevation of the mTOR level and a decrease in the Atg7 level are involved in the molecular mechanisms of DLB. Nevertheless, current data support the idea that the autophagy–lysosome pathway plays an important role in diseases that involve the aggregation of α-syn such as DLB and may provide potential research targets for the treatment of diseases, specifically the regulation of the autophagy–lysosome pathway.

1.3.2 FTD and Autophagy

Frontotemporal dementia (FTD) is a form of non-Alzheimer's disease dementia that is characterized by the localized atrophy of the frontal and anterior temporal lobes. Its clinical manifestations are progressive mental and behavioral changes, language disorders and cognitive dysfunction, sometimes accompanied by motor neuron disease or PD. Most cases of FTD are hereditary and involve autosomal dominant inheritance. Tau-positive FTD with parkinsonism is caused by a mutation in the tau gene.

Tau-negative FTD with ubiquitin-positive inclusion bodies is caused by a mutation in the progranulin (PGRN) gene. Interestingly, a large number of gene mutations associated with FTD, such as mutations in the CHMP2B (charged multivesicular body protein 2B) gene and the VCP (valosin-containing protein) gene, are also related to the process of autophagy. Mutations in the CHMP2B gene account for a relatively small proportion of cases of autosomal dominant FTD. The overexpression of C-terminal truncated CHMP2B in PC12 cell lines can interfere with normal autophagy processes and proteolysis. In addition, mutant CHMP2B has been found to cause autophagic vesicle aggregation, dendritic shortening and neuronal loss, possibly by affecting the fusion of autophagosomes and lysosomes. Other mutations associated with FTD are mutations in VCP/p97. Several studies have suggested that VCP may be involved in autophagy. VCP mutations (R155H or A232E) are associated with FTD, and the overexpression of VCP may cause autophagic vesicle aggregation and maturation dysfunction, suggesting that VCP plays an important role in the maturation of autophagosomes. LC3-II immunostaining has shown the aggregation of a large number of autophagosomes in mice expressing a R155H mutation in VCP, which indicates that the R155H mutation in VCP can cause abnormal autophagy in the brain. Although CHMP2B and VCP mutations account for a small proportion of FTD cases, the important roles of these genes in the autophagy pathway suggest that abnormal autophagy processes are involved in FTD.

1.3.3 VD and Autophagy

Studies have also found abnormalities in autophagic flux in VD. In rat models of vascular dementia, autophagy-related markers such as Beclin 1, cathepsin B and LC3 are all significantly increased. However, after treatment with wortmannin, an autophagy inhibitor, autophagy-related protein expression levels are not significantly increased, and hippocampal neuron damage is reduced. In atherosclerosis, in order to protect vascular endothelial cells and smooth muscle cells from damage, autophagy is activated to prevent the formation of vascular plaques. By activating autophagy, the autophagy inducer spermidine can prevent the accumulation of lipids and the formation of plaque necrosis. Therefore, the autophagy pathway may also be a potential target for the treatment of VD.

1.3.4 Prion Disease and Autophagy

As in other neurodegenerative diseases such as AD, in prion diseases and transmissible spongiform encephalopathies (TSEs), neurons die via programmed cell death, and the autophagic process may play a role in programmed cell death. Autophagy may coexist with apoptosis or may precede it, and the process may be induced by apoptotic stimuli. Liberski et al. extended these observations by using different models of

prion disease, including CJD and scrapie models. Interestingly, autophagic vacuoles are present in both of these models, which indicates that prion protein deposition may induce autophagic processes (Liberski et al. 2002). In addition, autophagy is also found in the optic nerve in experimental Gerstmann–Sträussler–Scheinker disease (GSS) in mice and experimental Creutzfeldt–Jakob disease (CJD) in hamsters (Liberski et al. 2017). A mixture of empty autophagic vacuoles and electron-dense lysosomal vesicles has been observed in these animal models in the late stage of disease. Dystrophic neurites filled with a mixture of mitochondria, empty autophagic vacuoles and electron-dense lysosomal vesicles are interpreted as the final stage of autophagy. Although the mechanisms by which prion proteins trigger autophagy have not been well described, autophagy may contribute to disease progression and may become a potential treatment target for prion diseases.

1.4 Conclusion

The aggregation of Aβ and tau proteins in the brains of patients with AD suggests that AD is a protein disease. During the pathogenesis of AD, there are deficits in the protein clearance function of the autophagy–lysosome pathway. The activation of autophagy in the early stage of AD and the impairment of autophagy–lysosome maturation in the late stage of disease can lead to the accumulation of autophagosomes, damage to neuronal nutrients, the inhibition of organelle circulation and neuronal degeneration. Fully understanding the function of autophagy is very important for the effective prevention and treatment of AD.

In other types of dementia, autophagy occurs wherever abnormal proteins accumulate. Targeting autophagy may effectively eliminate neuronal damage mediated by abnormal protein deposition. Future studies on the role of autophagy in neurodegenerative diseases may help to clarify this common mechanism and uncover the effect of abnormal protein aggregation in these diseases, and may also provide potential targets of interest for the treatment of these diseases.

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Chapter 2
Autophagy and Parkinson's Disease

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Abstract Parkinson's disease (PD) is the second most common neurodegenerative disease characterized by motor system dysfunction. The etiology of PD has been linked with aging, environmental toxins and genetic mutation, while molecular pathogenesis of PD includes various factors, such as impaired protein homeostasis, oxidative stress, mitochondria dysfunction, synaptic transmission impairment, calcium homeostasis imbalance, prion-like α-synuclein transmission and neuron inflammation. Autophagy is a conserved bulk degradation process to maintain cellular homeostasis. Impairment of autophagy has been reported to be involved in the pathogenesis of PD. Coding proteins of several PD-related genes, such as *SNCA, LRRK2, GBA, ATP13A2, VPS35* and *FBXO7*, are implicated in or affected by autophagy process. Furthermore, various pathogenic events during PD directly or indirectly interfere with the autophagy pathway, and dysregulation of autophagy has been observed in different neurotoxic PD models. Autophagy has been regarded as a potential therapeutic target for PD treatment. Indeed, modulations of autophagy-regulated genes (*BECN1* and *TFEB*) expression exerted neuroprotection against PD models, and various autophagy regulators, such as rapamycin, trehalose, lysosome modulators and other small molecule autophagy inducers, have displayed neuroprotective effects in experimental PD models. Taken together, autophagy dysfunction has been implicated in the pathogenesis of PD, and pharmacological modulation of autophagy may be a new therapeutic strategy for the PD treatment.

Keywords Autophagy · Parkinson disease · α-synuclein

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2.1 The Pathogenesis of Parkinson's Disease

Parkinson's disease (PD) is the most common motor system neurodegenerative diseases, and it was first reported by British doctor James Parkinson in 1817. The prevalence of PD is 1% in the elderly over 60 years old, and it reaches to 5–6% in the population over 85 years old. At present, the number of patients with PD in China exceeds 2.5 million. PD is clinically characterized by resting tremor, bradykinesia, muscle rigidity and gait abnormalities, accompanied by non-motor neurological symptoms such as olfactory disorders, depression, constipation and cognitive decline. The main pathological features of PD are the loss of the dopamine (DA) neurons in the middle brain *substantia nigra pars compacta* (SNpc), and the appearance of eosinophilic inclusion bodies called Lewy bodies (LBs) in the cytoplasm of residual neurons. The Lewy body is composed of α-synuclein (α-syn), ubiquitin, neurofilament proteins, synphilin-1, Parkin, UCHL-1 and other proteins. Autophagy is a process to degrade abnormal aggregated or misfolded proteins, as well as eliminate damaged cellular organelles, which are essential for maintaining neuronal homeostasis. Accumulated evidence suggests that autophagy is involved in the pathogenesis of PD. For example, abnormal autophagy-related structures and abnormally altered autophagy-related protein expression levels were observed in neurons in the *substantia nigra* of PD patients; PD pathological protein α-syn can be degraded by both selective and non-selective autophagy, and its overexpression or mutation leads to inhibition of autophagy; abnormal autophagy can be observed in toxin-induced PD animal models. In addition, several PD-related genes have been implicated in the autophagy–lysosome pathway. The relationship between PD and autophagy is discussed in detail in this section.

2.1.1 Pathogenesis of Parkinson's Disease

PD has been recognized and studied for nearly 200 years. Researchers commonly believed that genetic and environmental risk factors are involved in the development of PD. Although genetic factors are the cause of familial PD, the exact pathogenesis for the sporadic PD is unclear. At present, there are several theories to elucidate the PD etiology: aging theory, genetic susceptibility theory and environmental toxin theory. About 5–10% of PD manifests as family hereditary, and mutations in PD-related genes (such as *LRRK2*) can be found in some sporadic PD patients. There is a high incidence of PD in Israel, probably due to the prevalence of PD-related *LRRK2* and *GBA* mutations in Ashkenazi Jew. Some toxic substances such as MPTP can cause clinical symptoms of PD. The evidence suggests that both genetic factors and environmental toxins are involved in the pathogenesis of PD. However, it is still unclear how the genetic and environmental factors synergistically work to damage dopaminergic (DAergic) neuron. The following factors are the risk factors for PD.

2.1.1.1 Age

Neurons are highly differentiated cells whose number decreases with age. As people grow older, DA neurons in the *substantia nigra* gradually degenerate. The DA secretion, DA receptor expression, tyrosine hydroxylase (TH) and dopa decarboxylase (DDC) expression progressively decline. However, normal aging does not lead to symptoms of PD, unless when the number of neurons in the *substantia nigra* DA is reduced to less than 50%, and the striatum DA content falls below 80% of normal level. In addition, the function of the ubiquitin–proteasome system (UPS) and the autophagy–lysosome pathway (ALP) in the senile nervous system is gradually weakened, and abnormal protein aggregation is prone to occur. Recent studies have shown that patients developing PD at older age often show more severe movement symptoms and pathological markers. The level of movement symptoms, striatum damage and DA content are exacerbated with age.

2.1.1.2 Environmental Factors

Epidemiological survey results show that various environmental factors are involved in the occurrence of PD, including pesticide contamination, industrial heavy metals, such as manganese pollution, neurotoxins and organic solvents. Many reports suggest that pesticide contamination is a risk factor for PD. The occupational populations exposed to insecticides such as rotenone frequently have a high incidence of PD. Other pesticides such as permethrin, paraquat and organochlorines have been reported to induce PD. Heavy metal contamination has also been reported to be associated with PD, especially high dose of manganese exposure can cause manganese poisoning syndrome whose clinical symptom is parkinsonian syndrome. In 1982, several drug addicts suddenly developed symptoms of PD, and later found that the drugs they used were contaminated by MPTP. MPTP enters brain and can be metabolized by monoamine oxidase to highly toxic MPP^+, which induces oxidative damage by inhibiting mitochondrial oxidative chain complex I (mitochondrial complex I). Moreover, brain damage and drugs for the mental illness treatment can increase the incidence of PD. These evidences support that environmental factors are involved in the pathogenesis of PD.

2.1.1.3 Genetics

Although the majority of PD is sporadic, 5–10% of cases are familial. Epidemiological studies suggest that synergistic work of genetic and environmental factors is the most important cause of sporadic PD. Familial PD manifests as incompletely explicit autosomal or recessive inheritance. The clinical features of familial PD are early onset, ataxia, pyramidal system damage, and so on. However, the pathological features vary greatly, sometimes lack of Lewy bodies in the pathological examination. In the past ten or more years, multiple genes have been found to be associated with the

pathogenesis of PD through linkage analysis and gene mutation screening of familial PD. Among the genes, *Parkin*, *PINK*, *DJ1*, *ATP13A2*, *PLA2G6*, *FBXO7*, *DNAJC6* and *SYNJ1* belong to autosomal recessive inheritance, and *SNCA*, *LRRK2, VPS35, EIF4G1* and *GBA* are autosomal dominant inheritance. Mutation in the *LRRK2* can also be found in some sporadic PD, and the mutation rate in a special group can reach to 15 or 40% of PD patients. The discovery of these PD-related genes provides important information for further understanding of the pathogenesis of PD. However, how do these genes work in the pathogenesis of PD has not been fully elucidated.

2.1.2 Molecular Pathogenesis

The molecular pathogenesis of PD is unclear. Accumulated evidence suggests that abnormal protein folding and aggregation, abnormal protein degradation, oxidative stress, mitochondria dysfunction, imbalance of intracellular calcium homeostasis and synapse transmission dysfunction are closely associated with PD. A variety of pathological changes in the striatum and *substantia nigra* in PD patients suggest that multiple mechanisms are involved in the development of PD process. For instance, occurrence of abnormal protein aggregates suggests that the protein degradation pathway is weakened or impaired; increased lipid oxidative products and abnormal iron metabolism indicate the strong oxidative stress; dysfunction of mitochondrial respiratory chain complex I and damage to mitochondria DNA suggest mitochondrial dysfunction; reduced long-term potentiation (LTP) and long-term depression (LDP) indicate neurosynaptic pathway problem.

2.1.2.1 Impaired Protein Degradation Pathway

Neurons belong to highly differentiated cells, which rely on efficient protein clearance pathway to promote misfolded or damaged protein degradation and maintain cell homeostasis. As age grows, damaged or misfolded proteins continuously accumulate, disrupting the balance of protein accumulation and protein degradation in the brain. Moreover, the excessive protein accumulation causes proteolytic stress, which eventually leads to neuronal degeneration. There are two major protein degradation pathways in neuron, including ubiquitin–proteasome system and the autophagy–lysosomal pathway. The former promotes the degradation of soluble short-lived proteins in the proteasome by ubiquitin labeling, while the latter is responsible for transfer long-lived/abnormal aggregated proteins to lysosome by forming autophagosome. Lewy body is an important pathological feature of PD, and its main component is fibrotic α-syn. According to the studies, α-syn is degraded by both UPS and ALP, suggesting an important role for these two pathways in PD. Excessive α-syn can inhibit autophagy pathway, while the mutant α-syn (A53T, A30P) blocks the CMA pathway. In fact, a large body of evidence suggests that PD patients showed impaired UPS and ALP. A study found that PD patients have shown decreased proteasome

activity in the *substantia nigra* region, reduced expression of proteasome subunits, and decreased proteasome activity and UPS-associated protein expression in peripheral blood mononuclear cells. Abnormal proteasome function can also be observed in some toxin-induced PD animal models and α-syn transgenic mice. The role of ALP dysfunction in the pathogenesis of PD will be elucidated in detail in the following chapter.

2.1.2.2 Oxidative Damage

Oxidative damage is involved in neuronal dysfunction and death in both sporadic and familial PD patients. Researchers have detected obviously increased lipid peroxides, oxidative proteins and oxidative DNA, and reduced antioxidant like glutathione in the *substantia nigra* region of PD patients. Cellular oxidative equilibrium is decided by the reactive oxygen species (ROS) level and antioxidant activity. Once the balance is disturbed, oxidative damage would occur. DAergic neurons are particularly sensitive to oxidative stress due to its specific expression of tyrosine hydroxylase (TH) and monoamine oxidase B (MAO-B) which can accelerate ROS production. In addition, DAergic neurons in the striatum contain high concentration of iron, which can catalyze fenton reaction, converting superoxide ions and hydrogen peroxide into highly toxic hydroxyl radicals. The sensitivity of DA neurons to oxidative stress may be an important factor in the selective loss of DA neurons in the *substantia nigra* of PD patients. ROS source in DAergic neurons include: production during DA metabolism, mitochondrial oxidative respiratory chain dysfunction and inflammation-induced glial cell production. ROS can also cause cytotoxicity through other mechanism, such as promoting aggregation of α-syn and inhibiting proteasome function, except for oxidation of organelles, protein or DNA. Application of antioxidants is a strategy for PD treatment. MAO-B inhibitors and coenzyme Q10 are effective compounds in alleviating the progress of PD by reducing the oxidative damage of DA neurons.

2.1.2.3 Mitochondria Dysfunction

The first evidence linking mitochondria dysfunction to PD stems from a study of MPTP-induced PD. MPTP metabolite MPP^+ specifically inhibits DA mitochondrial oxidative respiratory chain complex I function, which causes mitochondria dysfunction, increase of free radicals and DA neuron death. In addition, rotenone which is used to induce PD model can also inhibit mitochondria respiratory chain complex I. The annonaceae contains natural mitochondrial respiratory chain complex I inhibitor, annonacin. Eating fruit or tea containing annonacin is found to be closely related to PD in Guadeloupe. According to the autopsy of the specimens from PD patients, mitochondria respiratory chain complex I in the *substantia nigra* and frontal cortex decreased 30%, and significantly increased oxidative damage in the subunits of the respiratory chain complex I. A study also found that about 25% of PD patients showed

decrease in respiratory chain complex I function, suggesting that some PD patients may develop systemic respiratory chain complex I dysfunction. Another study found that DA neuron-specific knockout of the respiratory chain complex I subunit Ndufs4 accelerated striatum DA metabolic rate, resulting in a decrease in axonal DA release. These results suggest that abnormal DA release may be the early change in mitochondria dysfunction in PD. As we know, mitochondria oxidative phosphorylation relies on function of a series of proteins encoded by genomic and mitochondria DNA, so mitochondria DNA has a large impact on mitochondrial function. In the DA neurons of the *substantia nigra*, mitochondrial DNA fragment deletion is also significantly increased with age, and the rate of mitochondria DNA mutation in DA neurons of PD patients is higher than that of the normal elder population. These mutations may be associated with the increased oxidative damage.

2.1.2.4 Synaptic Transmission Disorder

Stimulation-induced synaptic plasticity alterations, such as LTP and LTD, reflect the cellular mechanism of adaptive motor neurotransmission and memory learning. Inhibition of both LTP and LTD has been reported to cause motor dysfunction and cognitive impairment in PD patients. In fact, synaptic transmission at the end of the nigrostriatal pathway regulates the DA-induced LTP and LTD, and its efficiency in the striatum affects significantly on PD symptoms. The association between synaptic plasticity and PD is majorly from the study of animal PD models. Studies have found a series of changes in cell and synaptic activity in PD models. The dysfunction transport and assembly of N-methyl-D-aspartic acid (NMDA) receptors on striatum efferent cells can cause clinical symptoms in experimental animals. Moreover, studies on PD-related genes have revealed that multiple genes are involved in synaptic function regulation. For example, α-syn overexpression inhibits neurotransmitter release and affects the size of synaptic vesicle recovery pool; *DJ-1* and *PINK* knockout mice showed obvious synaptic dysfunction; *LRRK2* mutant transgenic mice also showed neurotransmitter transmission impairment. Recently, studies on the relationships between the function of presynaptic membrane vesicle trafficking and PD have gradually increased. Synaptic vesicles located on the presynaptic membrane of the striatum are responsible for isolating the surrounding material, encapsulating and transmitting neurotransmitters. Therefore, the dysfunction of synaptic vesicles has an important influence on the transmission of neurotransmitters. Many studies have found that PD-associated genes-coded proteins are involved in vesicle trafficking in the presynaptic membrane, such as α-syn, *LRRK2* and synaptojanin, all of which can cause abnormal DA release (Lotharius and Brundin 2002). More recently, research found that synaptic vesicle protein, glycoprotein 2C (SV2C), regulates DA homeostasis. In addition, the dysregulated SV2V expression can lead to some pathological features of PD.

2.1.2.5 Imbalance of Intracellular Calcium Homeostasis

Neurons maintain resting potential by concentration gradients of ions across the membrane, and then produce action potential to nerve impulse when the ion channel is open. Most neurons rely entirely on sodium channels to generate rhythmic electrical impulses, but DA neurons can also use the L-type calcium channel (Cav1.3) to increase calcium influx. As the calcium ion buffer system in DA neuron is weak, it results in increased calcium ion concentration and mitochondrial oxidative respiration activity. So, DA neuron is more susceptible to death induced by other toxic materials (Schapira 2013). The Cav1.3 calcium channel blocker, isradipine, can attenuate the rotenone- or MPTP-induced DA neuron loss in mice. Also, epidemiological investigations have shown that brain-permeable calcium channel blockers can reduce the risk of PD. Taken together, Cav1.3 may be an important target for PD treatment.

2.1.2.6 Prion-like α-Synuclein Transmission

α-Syn in neuron can be transmitted to various regions of brain by means of endocytosis and exocytosis along the axon system (Brundin et al. 2010). Therefore, α-syn aggregates could gradually increase over age and affect the function of various brain regions in the later stage. This hypothesis provides a good research direction for bowel constipation and symptoms of olfactory loss in the early stage of PD.

2.1.2.7 Neuron Inflammation

Catecholaminergic neurons in the brain of PD patients and DA neurons exposed to L-DOPA or activated microglia in vitro are more likely to express MHC class I, which make them more susceptible to be recognized by cytotoxic T cell and induce apoptosis (Cebrián et al. 2014). In addition, PD-related genes, such as *LRRK2*, are involved in the regulation of immune cells. α-Syn aggregates can induce innate and adaptive immune responses in PD models and patients. Conversely, neuroninflammation promotes the folding of α-syn (Ransohoff 2016). Recently, researchers have isolated T cells from PD patients and found that T cells can recognize α-syn polypeptides, which adds new evidence to the role of α-syn in stimulating immune response in PD (Sulzer et al. 2017). In some PD patients with prodromal symptoms, inflammatory response in olfactory system and intestines induce the production of large amount of α-syn and eventually forming polymers that can be hardly degraded.

2.2 The Roles of Autophagy in the Pathogenesis of PD

The evidence that autophagy participates in the pathogenesis of PD comes from the autopsy of PD patients. People observed the abnormal autophagy-related structures

in neurons in the *substantia nigra* of PD patients. In the followed studies, abnormal levels of autophagy-related protein in the *substantia nigra* and peripheral blood cells were observed in PD patients. In addition, abnormal autophagy can also be detected by different PD animal models, such as toxin-induced PD animal models, α-syn transgenic cells, mice and *Drosophila*. All the above evidence indicates that autophagy is involved in the pathological process of PD. In fact, many pathological mechanisms of PD are closely related to autophagy. For example, multiple products encoded from PD-related genes are involved in the autophagy regulation (Table 2.1). Oxidative damage and mitochondrial dysfunction can affect autophagy. UPS dysfunction can cause changes in autophagy levels. Alteration of mitochondrial membrane permeability induced by mitochondrial damage can activate mitochondrial autophagy. Lysosome function impairment caused by PD-related gene mutation is closely related to autophagy blockage. Multiple PD-associated toxins, like 6-OHDA, MPP^+, rotenone and paraquat, showed an effect on autophagy in the experimental model. The role of autophagy in the pathogenesis of PD is elucidated in detail in Fig. 2.1.

Table 2.1 The roles of PD-related genes in autophagy

Name	Inheritance patterns in PD	Influence on autophagy
α-Syn (SNCA)	Its mutation induces autosomal dominant PD	Autophagy substrate; its mutation inhibits CMA; excess or mutant α-syn inhibits autophagy
LRRK2	Its mutation causes autosomal dominant PD and sporadic PD	As CMA substrate; its mutation inhibits CMA
GBA	Its mutation increases PD susceptibility	Its mutation affects lysosomal activity and inhibits CMA
ATP13A2	Its mutation causes autosomal recessive inheritance	Act as lysosomal ATPase; its mutation causes lysosomal function inhibition
VPS35	Its mutation induces autosomal dominant PD	The mutants affected promote WASH complex aggregation and inhibit autophagy
PINK1	Its mutation induces autosomal dominant PD	Mitochondrial damage sensor; recruit *Parkin* to enhance mitophagy
Parkin	Its mutation induces autosomal dominant PD	Recruited to damage mitochondria; ubiquitinate outer membrane in mitochondria; promote mitophagy
FBXO7	Its mutation induces autosomal recessive PD	Work with *Parkin* and *PINK1* to promote mitophagy

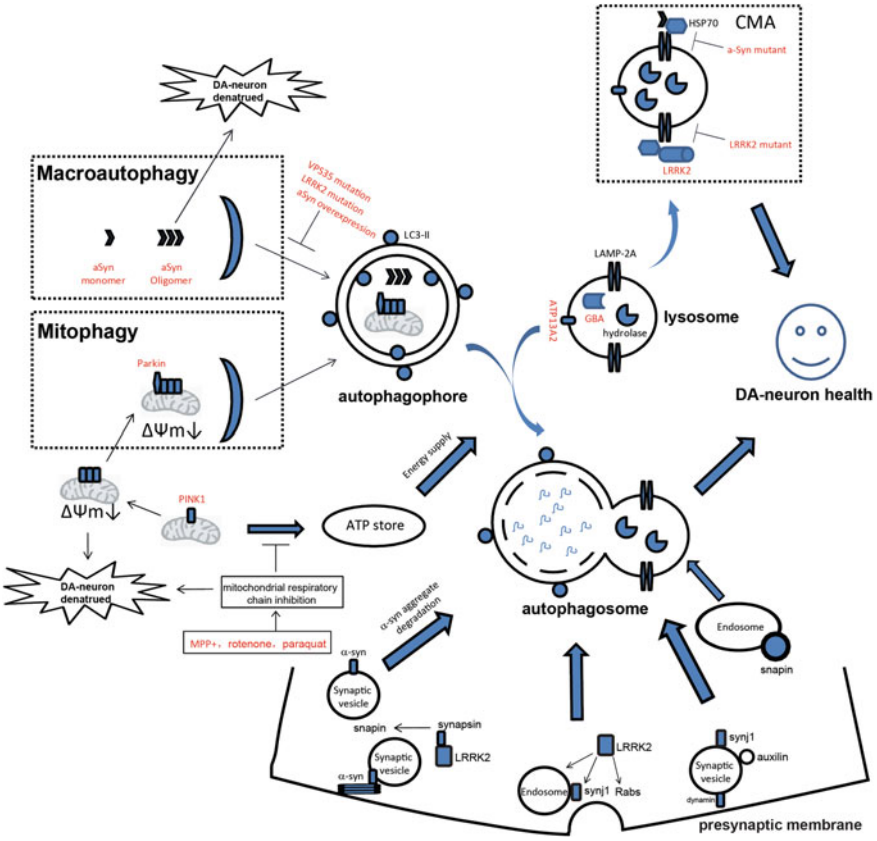

Fig. 2.1 The role of autophagy dysfunction in PD pathogenesis. In autophagy, macroautophagy, selective autophagy (especially mitophagy) and chaperone-mediated autophagy (CMA) are involved in the pathogenesis of PD. α-Syn is a substrate for macroautophagy and CMA. Mutated α-syn can inhibit CMA pathway, and α-syn overexpression can also inhibit macroautophagy. LRRK2 is a substrate for CMA, and its PD-associated mutants block the normal progression of the CMA pathway. A variety of PD-inducible toxins can cause mitochondria damage, leading to changes in mitochondrial membrane permeability. Impaired mitochondria recruit Parkin to the mitochondrial membrane to ubiquitinate proteins via PINK1, thereby promoting the fusion of damaged mitochondria and autophagosomes. Lysosomes is the final region of autophagy degradation. Some products encoding PD-related genes such as *ATP13A2* and *GBA* are related to lysosomal function. Mutation products can cause abnormal lysosomal function and affect the autophagosome, resulting in neuron death. A variety of PD-associated proteins are involved in the vesicle trafficking and autophagy regulation in the presynaptic membrane. For example, LRRK2 acts as a kinase to regulate a variety of trafficking-related proteins, regulating endocytosis and vesicle trafficking, and ultimately promoting autophagic degradation. Taken together, the proper function of the autophagic lysosomal system is of great significance for preventing neuronal death and alleviating pathological damage associated with PD

2.2.1 PD-Related Genes and Autophagy

2.2.1.1 *SNCA*

SNCA is the first identified PD-related gene. Specific site mutations in *SNCA* or an increase in the number of copies can directly lead to the development of early onset of familial PD. Recent studies have found that the gene polymorphism in the promoter region of the *SNCA* gene is associated with the dissemination of PD, further supporting the relationship between this gene and PD pathogenesis. The *SNCA* encodes a protein consisting of 140 amino acids, α-syn, with a relative molecular weight of 14 kDa. The amino acid sequence of α-syn is highly conserved and was previously thought not to be able to form a specific structure. However, recent studies have shown that α-syn acts as a polymer to form a spatial structure. The specific function of α-syn has not yet been elucidated, but some experimental evidence suggests that it is involved in the maintenance of synaptic function, which can regulate the size and circulation of synaptic vesicles, and it is involved in neuronal development and synaptic plasticity regulation. As α-syn is the main component of Lewy body in the brain of PD patients, it is closely related to the formation of Lewy bodies and the degeneration of DA neurons. α-Syn is prone to form aggregation, especially with a point mutation. Aggregated α-syn can form soluble oligomers, fiber precursors and mature fiber-like structures. It is generally believed that the oligomeric form of α-syn is highly toxic, and the Lewy body is a protective mechanism by which cells isolate α-syn to reduce toxic damage. When persistent aggregate formation exceeds the extent that cells can tolerate, apoptosis will be activated to accelerate cell death.

α-Syn can be degraded by UPS and ALP pathways. α-Syn is a substrate for UPS which can be transported to the proteasome for degradation via ubiquitination. However, excessive accumulation of α-syn can inhibit UPS activity, which impairs cell degradation. There is a crosstalk pathway between UPS and ALP system. When UPS is inhibited, the excess ubiquitinated products are transported to the autophagosome via binding with the autophagy receptors such as p62, NBR1 and NDP52. On the other hand, when the autophagy function is inhibited, obviously increased α-syn level can be observed. A recent study showed that deubiquitinating enzyme USP9X regulates the degradation of α-syn. Most deubiquitinated α-syn is degraded by autophagy, while ubiquitinated ones are degraded by proteasome. Studies on α-syn transgenic mice have shown that UPS system is the major pathway under normal condition for α-syn degradation, whereas ALP pathway is activated when α-syn is excessively aggregated. It is generally believed that α-syn monomer is degraded by UPS, while oligomer or fiber aggregate is mainly degraded by ALP. Due to the high toxicity of oligomeric α-syn, ALP is considered as an important mechanism to promote degradation and by this way to maintain cell homeostasis. Many researchers are now looking for new strategies to activate ALP pathway for PD treatment.

Autophagy can be divided into three classes: microautophagy, macroautophagy and chaperone-mediated autophagy (CMA). α-Syn is closely related to macroautophagy and CMA. CMA refers to the lysosomal degradation process in which the

substrates are transported by a chaperone protein. In CMA, the amino acid sequence in its substrate requires a conserved recognition region such as KFERQ or other highly structurally similar sequences (Kaushik and Cuervo 2012). Coincidentally, α-syn contains a sequence (95VKKDQ99) which can be recognized by HSP70 and transported to the lysosomal surface to bind with LAMP2 for degradation. Wild-type α-syn in neuronal cell lines and primary neurons can be degraded by CMA, but not mutant α-syn without the 95VKKDQ99 sequence (Cuervo et al. 2004). Moreover, CMA-associated HSP70 and LAMP2 are significantly reduced in the *substantia nigra* and amygdala in PD patients, indicating that CMA dysfunction is involved in the pathogenesis of PD. Also, A53T mutant α-syn shows strong binding ability to LAMP 2 accompanied by CMA inhibition with impaired lysosome degradation. CMA is also linked to the degradation of key factors in cell survival. Myocyte enhancer factor 2D (MEF2D) is an important protein for neuron survival, and it can be degraded by CMA. Both wild-type and mutant α-syn can inhibit the MEF2D degradation, causing abnormal MEF2D activity and thus leading to cell death. In addition, increased MEF2D can be detected in both α-syn transgenic mice and in the PD patients, suggesting that abnormal elevation of MEF2D due to CMA inhibition may be a potential pathological mechanism for PD. Studies have found that α-syn can inhibit the early stage of autophagosome formation by affecting the Rab1a function (Winslow and Rubinsztein 2011). However, other studies thought that α-syn does not affect the early stage of autophagosome formation, but the degradation of autophagosomes. A recent study revealed that both wild-type and mutant α-syn overexpression can inhibit autophagy in PC12 cells by its interaction with beclin1 by binding to HMGB1. Recently, a study found that α-syn can be secreted extracellularly, and there is evidence that the secretory process is dependent on normal autophagy mechanism which is different from the classical endoplasmic reticulum-Golgi secretion pathway. A recent study also found that autophagy inhibition promotes extracellular α-syn secretion in the form of autophagosome-like exosomes rather than vesicles (Minakaki et al. 2018). However, the specific role of autophagy in the secretion process of α-syn needs further study. In summary, the excessive aggregation of α-syn is dependent on autophagy, but they can also inhibit autophagy under certain conditions. The contradiction plays an important role in the PD development. So, elucidating the molecular mechanism that how α-syn modulates autophagy is valuable for designing small molecule compounds for autophagy restoration.

2.2.1.2 *LRRK2*

The leucine-rich repeat kinase 2 (*LRRK2*) gene mutation is currently the most important genetic factor in PD pathogenesis. The clinical phenotype of PD patients associated with *LRRK2* mutations are similar to that of sporadic PD. The symptoms include DA neuron degeneration and the Lewy bodies formation, except for typical motor neurological dysfunction. The prevalence of *LRRK2* mutation can reach to 20–30% of the total PD patients in specific population, and *LRRK2* mutation can account for 3–7% of the hereditary PD worldwide. Until now, there are more than

50 *LRRK2* mutations that have been identified, and G2019S mutation can be found in about 2% of total PD patients. Compared to other population, there are fewer PD patients associated with *LRRK2* mutations in Asia. *LRRK2* gene encodes LRRK2 consisting of 2527 amino acids, and the molecular weight is approximately 280 kDa. LRRK2 protein contains at least a kinase domain, a GTPase domain, a COR domain, a WD40 domain and a leucine-rich repeat domain. However, the *LRRK2* function is still unclear, but some evidence suggests that it is involved in the endocytic process of synaptic vesicles, neurotransmitter release modulation and regulation of synaptic structures. G2019S is the most important PD-associated LRRK2 mutation site, whose mutation increases the LRRK2 kinase activity, causing a decrease in neurites in primary cultured neurons. The results suggest that increased LRRK2 activity may affect the neuron growth, and is involved in the pathogenesis of PD. Many researchers are looking for the inhibitors and the substrates for LRRK2 kinase, hoping to provide new ways for the treatment or early diagnosis of PD patients with LRRK2 mutations. Recent studies have identified some LRRK2 substrates and found that increased LRRK2 kinase activity promotes phosphorylation of Rab10, a LRRK2 substrate, causing mitochondrial protein and lysosomal function dysfunction (Di Maio et al. 2018). Rab35, another LRRK2 substrate, has also found to induce α-syn overexpression (Bae et al. 2018).

Wild-type LRRK2 is degraded by the UPS pathway, while mutant LRRK2 relies on the autophagy pathway for its degradation. A recent study found that wild-type LRRK2 can also be degraded by CMA pathway, while PD-associated *LRRK2* mutants inhibit CMA function, leading to the accumulation of other CMA substrates, such as α-syn (Orenstein et al. 2013). *LRRK2* mutants inhibit CMA mainly through its binding with LAMP2 on the lysosome surface to affect the transport function. In addition, many studies have shown that *LRRK2* is closely related to autophagy. The accumulation of autophagy substrates and α-syn in the kidney of *LRRK2* knockout mice suggests that the autophagy is inhibited. However, autophagy inhibition in the nervous is not observed. So, whether LRRK2 has different regulatory effects on autophagy in different tissues still need further study. Studies have shown that different *LRRK2* mutations can activate or inhibit autophagy under different cellular conditions. People found that autophagic vesicles were increased in the cell bodies of neurons which were transfected with the G2019S mutation, accompanied by neurite outgrowth inhibition. This phenotype can be alleviated by silencing Atg5 or Atg7, and enhanced by treatment of autophagy inducers such as rapamycin, indicating that autophagy is involved in the nerve growth inhibition in the G2019S mutation. Meanwhile, study also found that p62 and LAMP1 were significantly lower in G2019S mutation PD patient than those in spontaneous PD patients, suggesting that *LRRK2* mutant induced a more pronounced change in the autophagy–lysosome process. Although LRRK2 is mostly located in the cytosol, approximately 10% of LRRK2 is located on the mitochondrial membrane, suggesting its potential association with mitochondrial function. Fibroblasts isolated from PD carrying the G2019S mutation showed reduced mitochondrial membrane potential and cellular ATP content. It has also been reported that LRRK2 G2019S mutant binds directly to Drp1 and phosphorylates its Thr595 site, causing excessive mitochondrial separation. The degradation

of mitochondria through autophagy may be observed in mouse cortical neurons expressing LRRK2 G2019S and R1441C mutants, possibly indicating the association with the cellular calcium homeostasis. In addition, recent studies have shown that *LRRK2* G2019S mutation can induce mitochondrial DNA damage in a kinase activity-dependent manner. The evidence suggests that *LRRK2* may be involved in the autophagy function regulation during the pathogenesis of PD.

2.2.1.3 *GBA*

Homozygous mutations in *GBA* gene can cause lysosomal disease and Gaucher disease. Patients with homozygous mutation of GBA often exhibit PD-like clinical symptoms and DA neuronal degeneration, while its heterozygous mutations are the most common risk factor of PD. The *GBA* gene encodes a lysosomal glucocerebrosidase whose primary function is to catalyze the glucosylceramide to glucose and ceramide. Homozygous or heterozygous mutations in *GBA* inhibit GBA protein function, leading to aggregation of glucosyl ceramide in neuron and inhibition of lysosomal degradation. Previous studies have reported that lysosomal function inhibition can cause α-syn aggregation and form oligomers, which would further affect the GBA transportation from the ER to lysosomes. The positive feedback mechanisms involved eventually induced neuron death. A recent study showed that the GBA protein function is impaired in the early stages of PD, but it does not increase with the progression of the disease via analyzing the brain samples from PD patients. It may not be the direct reason for the neuron death. The authors speculate that impaired GBA function can disturb the CMA pathway, leading to DA neuron degeneration via increasing α-syn aggregation and decreasing ceramide. It has also been reported that N370S *GBA* mutation greatly increases the cholesterol accumulation, thus altering autophagosome lysosomal function and increasing apoptosis (García-Sanz et al. 2018).

2.2.1.4 *ATP13A2*

ATP13A2 mutation can cause autosomal recessive inheritance of DA-responsive PD syndrome (Kufor-Rakeb syndrome, KRS). The clinical manifestations of KRS vary widely and are dependent on the mutation site. *ATP13A2* encodes a lysosomal ATPase involved in the selective transport of cations on inner or outer side of the membrane. Studies on *Caenorhabditis elegans* have found that *ATP13A2* homologous proteins protect cells from manganese ions and other heavy metal ions that cause toxic damage. Interestingly, ATP13A2 attenuated the α-syn misfolding causing damage to *C. elegans* and primary cultured PD neurons, indicating that these two PD-related genes are pathologically associated with PD (Gitler et al. 2009). It has been reported that *ATP13A2* can help cells fight against a variety of cellular stress-induced injuries, including mitochondrial respiratory chain complex I inhibition, oxidative damage, metal ion toxicity and proteasome stress. Lysosome function is generally impaired in

KRS patient-derived fibroblasts and *ATP13A2* deletion cell lines, including decreased lysosomal membrane stability, lysosomal acidification process, lysosomal substrate degradation inhibition and impaired autophagosome degradation. All these phenotypes can be reversed by overexpression of wild-type *ATP13A2* (Dehay et al. 2012). In *ATP13A2*-deficient neurons, silencing *SNCA* expression can alleviate cytotoxicity, and loss of *ATP13A2* function leads to the inhibition of mitophagy. A recent study revealed that *ATP13A2* is required for autophagosome maturation by regulating HDAC6 activity to promote autophagosome–lysosome fusion. In the meantime, *ATP13A2* is closely related to another novel PD-related gene *SYT11* (synaptotagmin11) by GWAS analysis. *ATP13A2* can regulate *SYT11* at both transcriptional and post-translational levels, and the *ATP13A2*-regulated autophagy is dependent on the process involving *SYT11* (Bento et al. 2016).

2.2.1.5 *VPS35*

VPS35 mutation can cause autosomal dominant inheritance and DA-responsive PD. The pathogenic 1858G > A (protein D620N) was found in multiple PD families, as well as in one sporadic PD patient. *VPS35* encodes a subunit of retromer complex whose primary role is to facilitate the reverse transport of endosomes to the Golgi complex, thereby promoting the recycling of certain membrane proteins or SNARES complexes. Retromer is also involved in the Wiskott–Aldrich syndrome protein and SCAR homolog complex (WASH) centralization. D620 mutation reduces its interaction with WASH complex, which shows significantly inhibited autophagy progress. Although the mechanism has not yet been well elucidated, the effects of the SNARE complex and ATG9 function may be involved. In addition, mutation in *VPS35* also affects LRRK2 activity and promotes the phosphorylation of related-Rab proteins in PD patients.

2.2.1.6 *FBXO7*

FBXO7 gene mutation was first discovered in the early-onset form in PD patients with autosomal recessive inheritance. Clinical studies have shown that PD patients with *FBXO7* mutations exhibit early clinical symptoms of PD, such as sensitive to levodopa, but some patients also developed mental retardation. FBXO7 protein is localized in mitochondria and interacts with Parkin and PINK1 to maintain mitochondrial function and promote *Parkin*-regulated mitophagy (Burchell et al. 2013). *FBXO7* protein function impairment results in reduced UPS degradation system, and leads to neurodegeneration.

2.2.2 *Oxidative Damage, Mitochondrial Dysfunction and Autophagy*

The brain is the organ that consumes the most oxygen in the body, and the neurons are strongly dependent on oxygen molecules. Neurons lack a glycolytic metabolic pathway that relies primarily on mitochondrial oxidative phosphorylation to produce energy, so oxygen and mitochondria are essential for maintaining normal neuronal metabolism. Mitochondrial oxidative phosphorylation provides the energy required for basic cellular activities of neuron, including UPS and ALP degradation of protein aggregation. Mitochondrial oxidative phosphorylation is accompanied by the production of free radicals, and DA neurons are more likely to cause oxidative damage due to the higher DA concentration and iron, so oxidative stress has been considered as one of the important pathological mechanisms of PD.

ATP is mainly produced by mitochondrial oxidative phosphorylation in neuron. The oxidative phosphorylation relies on the cooperation of mitochondrial respiratory chain complex I-IV, in which the free radicals are continuously produced, increasing the oxidative stress in the cell. Superoxide anion is the most important reactive oxygen species produced by the mitochondrial respiratory chain. It can be converted to hydrogen peroxide by superoxide dismutase or manganese superoxide dismutase, and then further reduced to water by peroxidase. In the presence of iron, hydrogen peroxide can form a highly toxic hydroxyl radical by fenton reaction, causing a series of cell damage, including lipid peroxidation in cell membrane, protein peroxidation, DNA oxidative damage and so on. The oxidative damage ultimately leads to neuron death. PD patients usually show high iron concentration in the *substantia nigra* region, and the DA neurons also display excessive oxidation, which makes the DA neuron withstand a lot of oxidative stress load. Once the antioxidative system is unable to defend against oxidative stress, it will cause cell damage or even cell death. In fact, studies have found that PD patients have increased oxidative stress in the nigra–striatum pathway (Bosco et al. 2006), and the level of antioxidant GSH is significantly reduced. Oxidative damage to various proteins can be detected in brain tissue in autopsy specimens of PD patients, including the appearance of excessive oxidation in α-syn before the clinical symptoms of PD.

Mitochondrial respiratory chain complex I is the main source of reactive oxygen species. People have long observed approximately 30–40% reduction in mitochondrial respiratory chain complex I function or protein levels in the *substantia nigra* from autopsy specimens of PD patients. Reduced complex I was also detected in the mitochondria isolated from the frontal cortex of PD patients. A decrease in the number of complex I was found in the stratum, cerebral cortex and various peripheral tissues of PD patients. The reduction of complex I is accompanied by an increase in the oxidation of its catalytic subunit protein, indicating that the peroxidation of the catalytic subunit may be an important cause for the decrease in the function of complex I. Complex I dysfunction also disrupts the oxidative phosphorylation in addition to decreasing the energy supply. Furthermore, increase of oxidative coenzyme Q10 and 8-hydroxy-2′-deoxyguanosine was also detected in the cerebrospinal

fluid of PD patients, suggesting that mitochondrial oxidative damage is involved in the pathogenesis of PD. Also, studies have shown that antioxidants can inhibit the development of PD to some extent. For example, taking high dose of coenzyme Q10 can improve motor function in early stages of PD patients. Mitochondrial oxidative stress is found in the cell damage caused by overexpression of catecholamines and insecticide exposure. Therefore, mitochondrial dysfunction and oxidative damage in course of PD are likely to be mutually causal, and the formation of a vicious circle eventually leads to the death of DNA neurons.

The encoded products from multiple PD-related genes are also closely related to mitochondrial function. α-Syn accumulates in the mitochondria of the *substantia nigra* in PD patients, and A53T mutant mice also shows mitochondria accumulation with mitochondrial damage and dysfunction of respiratory chain complex IV. Mutations in *Parkin*, *PINK1* and *DJ-1* can cause autosomal recessive PD, and these mutations can lead to abnormality of mitochondrial structure, function and transport. Parkin recognizes damaged mitochondria and mediates mitophagy to clear and circulate the damaged mitochondria. The interaction of Parkin and PINK1 enhances mitochondrial homeostasis and autophagy degradation. DJ-1 is a sensor of oxidative proteins and redox reactions that promote cell survival by enhancing cellular antioxidant capacity and activating PI3K/Akt signaling pathway. In addition, many toxins that can induce PD, such as 6-OHDA, MPP^+ and rotenone can cause mitochondrial damage and oxidative stress via inhibition of specific mitochondrial respiratory chain complex.

Both endogenous and exogenous reactive oxygen species are involved in the autophagy regulation. Starvation, nerve growth factor deficiency, rapamycin, tumor necrosis factor and other stress-induced autophagy require the involvement of reactive oxygen species. Nerve growth factor deficiency can cause early reactive oxygen species bursts and later accumulation of reactive oxygen species, accompanied by autophagosome aggregation. The antioxidant N-acetylcysteine can reduce the production of reactive oxygen species and autophagy. More direct evidence supporting the regulation of reactive oxygen species in autophagy is that ROS is involved in the cysteine vulcanization modification at position 81 of ATG4, a modification that is important for ATG4 processing LC3-l to LC3-ll, and LC3-ll recycling (Scherz-Shouval et al. 2007). HMGB1 is an inflammation-associated protein, and studies have shown that HMGB1 can promote autophagy via binding to beclin1. ROS plays an important role in the intramolecular sulfidation modification of HMGB1. N-acetylcysteine inhibits the intramolecular sulfation modification of HMGB1, attenuates the binding of HMGB1 to beclin1 and inhibits autophagy. In addition, oxidative damage can directly activate the transcription factor Tp53, which regulates the expression of autophagy-related proteins and enhances autophagy levels. The evidence suggests that ROS can be involved in the autophagy regulation at both post-translational and transcriptional levels. Interestingly, autophagy can in turn regulate cellular oxidation levels. The process of autophagy inhibition is accompanied by a disturbance of material energy circulation, impaired mitochondrial degradation, increased oxidative damage, ubiquitination of autophagy substrates and autophagy

receptors accumulation, such as p62. p62 can bind to Keap1, which release the antioxidant transcription factor Nrf2 to enter into the nucleus to initiate the expression of a series of antioxidant proteins. Collectively, there is a complex and fine adjustment between the active oxygen species and autophagy.

2.2.3 Selective Autophagy

Autophagy can be selective, in terms of cargo recognition and degradation. Autophagy can selectively clear substrates according to the different cargoes, such as misfolded proteins (aggrephagy), impaired mitochondria (mitophagy), peroxisome (pexophagy), endoplasmic reticulum (reticulophagy), ribosome (ribophagy) and pathogen (xenophagy). The selectivity of autophagy is dependent on the interaction of autophagy substrates with autophagy receptors, such as chaperones and other common autophagy-related proteins. Although the specific molecular mechanism is still unclear, selective autophagy has attracted extensive attention to study its mechanism in different diseases and autophagy-related intervention due to the specificity.

2.2.3.1 Aggrephagy

After proteasome activity is inhibited, ubiquitinated proteins tend to form aggregate, which are required to be degraded via autophagy (Kirkin et al. 2009). This is the major reason that most of the proteasome pathway-related components are often involved in the autophagy regulation. The process to clear protein aggregates via autophagy is called aggrephagy. This process is accompanied by ubiquitination of protein aggregates and recognition by the common autophagy receptors such as p62 and NBR1. In addition, more and more receptors or adaptors are being discovered; for example, ALFY, which binds to LC3 (Lystad et al. 2014), and WDR81, which binds to p62 and LC3 to regulate aggrephagy.

In PD, the toxic α-syn is ubiquitinated by E3 ligase SIAH (seven in absentia homolog) and can be degraded by proteasome. However, when the ubiquitinated α-syn has been deubiquitinated by the ubiquitin-specific protease 9X (USP9X), it is degraded via autophagy. In addition, α-syn can also be deubiquitinated via USP8, and the lysosomal degradation of α-syn is increased when USP8 activity is impaired and K63 conjugation increased. Therefore, the whole process of aggrephagy is mainly regulated by various ubiquitination, and the specific mechanism has not been elucidated. As α-syn has long been regarded as the therapeutic target for PD, aggrephagy may be a promising target for PD treatment. Accumulating studies have reported that α-syn clearance can be degraded by autophagy, but the specific mechanism is unclear. Exogenous fibrillar α-syn can induce autophagy in microglia, and can also recruit TANK-binding kinase 1 (TBK1) and optineurin (OPTN) for selective autophagy. Moreover, post-transcriptional modifications are also involved in the regulation of

autophagic degradation of α-syn. For example, it has been reported that excessive O-GlcNAcylation inhibits α-syn clearance by autophagy and has a destructive effect on neurons.

2.2.3.2 Mitophagy

As an energy factory, mitochondria are subjected to a great metabolic stress and are prone to organelles damage. There is a dynamic balance in the degradation of mitochondrial production in cells: neonatal mitochondria gradually replace aging or damaged mitochondria, while senescent or damaged mitochondria are cleared by cellular degradation mechanisms. When the mitochondrial degradation pathway is disrupted, the damaged mitochondria will accumulate in the cell, inducing apoptosis. Therefore, efficient mitochondria clearance is essential to maintain cell homeostasis. In normal conditions, damaged mitochondria are mainly degraded via macroautophagy and microautophagy, and this process is called mitophagy (Youle and Narendra 2011). Damaged mitochondria accumulation has been observed in the *substantia nigra* in the autopsy specimens of PD patients. In addition, damaged mitochondria and its aggregation can also be observed in various cell and animal models of PD. All these findings suggest that mitochondrial degradation problem is involved in the pathogenesis of PD.

The studies about the relationship between mitophagy and PD have made a great progress in recent years. Studies have found that α-syn can produce cytotoxicity and induce mitophagy. In addition, seeding recombinant fibrillar α-syn (PFF) in the neuronal cell line or primary neural cells can induce insoluble fibril form of α-syn. This fibril form of α-syn showed phosphorylation at the S129, which coincided with the results of the Lewy bodies in clinical PD. The formed insoluble α-syn fibril can disrupt autophagy, resulting in vesicular trafficking disorders, synaptic dysfunction (Volpicelli-Daley et al. 2011) and neuron death (Volpicelli-Daley et al. 2011). More recently, some researchers have discovered a phosphorylated α-syn fibril with greater cytotoxicity. It can induce mitochondrial toxicity, mitochondrial division, energy stress and mitophagy. Moreover, studies have shown that multiple PD-related genes play an important role in mitophagy. Mutations in the *Parkin*, *DJ-1* and *PINK1* genes are associated with autosomal recessive, early-onset familial PD. *Parkin*, *PINK1* and *DJ-1* act as ubiquitin ligases in a complex form in cell. Knocking out *Parkin* and *PINK1* genes in *Drosophila* can lead to similar phenotypes such as muscle tissue degradation, cell death and mitochondrial dysfunction (Clark et al. 2006). Overexpression of *Parkin* attenuated the *Drosophila* phenotype caused by *PINK1* knockout, but overexpression of *PINK1* didn't show similar effects. The results indicate that *Parkin* and *PINK1* are involved in the same functional pathway in cell, and that *PINK1* acts upstream of *Parkin*. Recent studies have shown that *Parkin* and *PINK1* are important regulators of mitophagy in mammalian cells. *Parkin* S65A mutation exhibit selective motor disorders, but they are not associated with neurodegeneration or mitophagy in nigrostriatal.

PINK1 is a protein localized at outer mitochondrial membrane, and widely expressed in various tissues with a relative molecular weight of 64 kDa. PINK1 comprises a serine/threonine kinase domain, at least one transmembrane region and a mitochondrial out membrane anchoring sequence at its carbon terminus. In normal conditions, PINK1 degrades rapidly, maintaining a low level. In current studies, PINK1 has been reported to be cleaved by mitochondrial proteases into a 60 kDa fragment on the mitochondrial membrane and then cleaved by PARL protease to generate a 53 kDa fragment. The 53 kDa fragment can be released into cytosol and be degraded rapidly by protease. Therefore, the PINK1 level in cells is maintained low. Once the mitochondrial membrane potential has been decreased, the proteolytic process of PINK1 is inhibited, resulting in rapid aggregation of PINK1 on the outer membrane of the mitochondria. Aggregated PINK1 will rapidly recruit Parkin to the mitochondrial outer membrane to work. Parkin is a cytoplasmic protein with a relative molecular weight of 52 kDa. It contains a ubiquitin-like domain and a RING domain. Its main function is to promote the degradation of ubiquitinated substrates as E3 ubiquitin ligase. PINK1 and Parkin interact intracellularly and can phosphorylate or ubiquitinate each other. When Parkin is recruited to the mitochondrial outer membrane by PINK1, the mitophagy is initiated by a series of protein ubiquitination processes. PINK1 and Parkin may activate mitophagy through several molecular mechanisms.

PINK1 and Parkin Recruit Autophagy-Related Proteins to Mitochondria

In yeast, mitophagy can be activated by nitrogen restriction or by rapamycin treatment. The damaged mitochondria can be transported to the autophagosome for degradation via the work of receptor Atg32 on the mitochondrial outer membrane, which binds to the autophagosome protein Atg8 and the autophagy receptor protein Atg11. This process can be regulated by MAPK kinase. Although no homologous protein of Atg32 has been identified in mammalian cells, several mitochondrial proteins in outer membrane have been elucidated to mediate binding of mitochondria and autophagosomes. Nix is another receptor to promote mitophagy by binding to p62 during reticulocytes maturation. Overexpression of Nix repairs mitophagy in fibroblasts from *PINK1* or *PARK2* mutant PD patients induced by CCCP. FUNDC1 promotes mitophagy by interacting with LC3 in an anoxic environment. In addition, other mitochondrial proteins in the outer membrane with a LIR structure such as FKBP8 may also recruit LC3 to induce Parkin-dependent mitophagy (Bhujabal et al. 2017). P62-mediated mitochondra aggregation, and the mechanism for p62 recruitment to the aggregated mitochondria may be related to ubiquitination of mitochondrial outer membrane proteins, because p62 can specifically bind to mitochondrial outer membrane ubiquitinated proteins and promote its fusion to autophagosome. Parkin is recruited to the mitochondrial outer membrane, which can ubiquitinate the outer membrane proteins such as VDAC1, and recruit p62 and LC3 to promote mitophagy. In addition, Parkin can induce ULK1/FIP200 punctate aggregation and ATG9A localizes to mitochondria, then activates downstream of autophagy.

PINK1 and Parkin Enhance Mitochondria Fission

Mitochondrion is a highly maneuvering organelle that maintains efficient fusion and fission to stabilize the homeostasis of mitochondrial network. Mitochondria can separate healthy mitochondria from damaged one through fission. Excessive dilation of mitochondria can be observed in *Drosophila* cells without *PINK1* and *Parkin*, possibly due to the impaired mitochondrial fission mechanism. This phenotype can be reversed by overexpression of mitochondrial protein DRP1 or by interference with the expression of the mitochondrial fusion protein OPA1 or dMFN, indicating that the PINK1/Parkin pathway promotes mitochondrial fission. In mammalian models, overextended mitochondria can be observed in hippocampus and DA neurons from *PINK1* knockout mice, while overexpression of PINK1 or Parkin results in the increased mitochondria number and reduced volume. In addition, a decreased mitochondrial membrane potential can enhance the inhibition of ubiquitination and degradation of mitochondrial proteins MFN1 and MFN2. These results suggest that PINK1 and Parkin may enhance the mitochondrial fission by promoting the degradation of MFN1, thereby accelerating the mitophagy.

PINK1 and Parkin Affect Mitochondrial Transport

In neurons, mitochondria are transported along tubulin by the binding of mitochondrial outer membrane anchoring protein Miro plus its binding protein Milton with forward transport kinesins or reverse transport dynein. A screening study found that Miro and Milton form a complex with PINK1. Studies on *Drosophila* showed that Miro interference can reverse the phenotype of the *PINK1* mutant, while Miro overexpression can induce a phenotype which is similar to PINK1 interference, accompanied by enhanced mitochondrial motility. Results have shown that a decrease in mitochondrial membrane potential is accompanied by aggregation of Parkin on the membrane and a decrease in mitochondrial motility. These results suggest that *PINK1* and *Parkin* may contribute to mitochondrial transport and promote mitophagy by promoting ubiquitination and degradation of Miro.

In addition to Parkin-dependent mitophagy, recent reports have found that if cardiolipin is exposed to the mitochondrial surface, more α-syn aggregates and mitochondrial fragments appear in the *SNCA*-mutated neurons near the mitochondrial membrane. The cardiolipin around the mitochondria recruits LC3 to the mitochondria and induces mitophagy (Ryan et al. 2018).

The evidence suggests that no matter for the clearance of excessive or mutatant α-syn aggregates in Lewy body. or for the clearance of damaged mitochondria, selective autophagy plays an important role in the pathogenesis of PD.

2.2.4 Lysosome Function and Autophagy

Lysosome is a place to digest proteins, lipids and organelles for maintaining the energy cycle of substances in cells. In autophagy, after the autophagosome is formed, it needs to be fused with lysosome to complete the degradation of its contents. Therefore, the normal function of lysosome is very important for the autophagy-dependent degradation process. Accumulated autophagosomes, reduced lysosomal numbers and decreased lysosomal proteins such as LAMP1 and HSP70 are observed in neurons in the *substantia nigra* of PD patients, suggesting a lysosome dysfunction. Fibroblast-derived DA neurons that isolated from the skin of PD patients also have shown lysosome dysfunction and autophagosome degradation inhibition. In the MPTP-induced mouse PD model, lysosome dysfunction was observed before cell death and caused autophagosome degradation inhibition. In this model, administration of rapamycin can reactivate the autophagosome lysosome pathway, resulting in increased lysosomal count, reduction in autophagosome accumulation and alleviation of DA neuron death.

Recent studies on familial PD-related genes have further supported the role of lysosomal dysfunction in the pathogenesis of PD. PD associated *SNCA* and *LRRK2* mutants can inhibit the CMA pathway and affect lysosomal function by sequestering LAMP1 on the lysosome membrane. *GBA* is a glycolipidase found in lysosomes. PD-associated *GBA* mutants losing the normal function has showed glucosyl ceramide aggregation in neurons and inhibited lysosome function, resulting in impaired α-syn degradation and oligomer forming. The latter further interferes with the transport of *GBA* protein from ER to lysosomes, ultimately leading to the neuron death. Loss of *ATP13A2* function is also associated with PD syndrome with lysosome dysfunction. *ATP13A2* mutation in neurons has shown reduced lysosomal membrane stability, impaired lysosome substrate degradation, accumulation of autophagosome and elevated levels of α-syn. This suggests that impaired lysosome function-induced autophagy inhibition is involved in the pathogenesis of PD. In addition to the study of PD-related genes in lysosome function regulation, large number of mutations in lysosomal storage disease-related genes have recently been identified in PD patients, further confirming the importance of lysosome function in PD (Robak et al. 2017).

Recent studies have found that the reduced activity of glucocerebrosidase, lysosome dysfunction and increased α-syn are induced by accumulation of oxidized DA which is stimulated by mitochondrial oxidative stress in sporadic and familial PD patients (Burbulla et al. 2017). The results suggest that the accumulation of oxidized DA may be an important link from mitochondrial disorders to lysosomal dysfunction.

2.2.5 Synaptic Vesicle Trafficking and Autophagy

Synaptic dysfunction has long been recognized as one of the important markers of many neurodegenerative diseases (Auffret et al. 2010). The function of the presynaptic membrane is to release various neurotransmitters through extracellular secretion, achieving neurotransmission. Therefore, dysfunction of the presynaptic membrane will lead to impaired neurotransmitter transmission process. When the secretory process is completed, synaptic vesicles and various proteins will be recycled through endocytosis. The whole process requires the coordinated cooperation of multiple proteins in the cell, including many autophagy-related proteins, such as clathrin that assists the formation of PI (4,5) P2, endophilinA1 and synaptojanin1 that binds to endophilinA1. Many studies suggest that autophagosomes also exist at the presynaptic membrane and can return to the cell body for degradation function with the help of dynein (Maday and Holzbaur 2014). Although the specific mechanisms involved in synaptic vesicle trafficking and autophagy lysosome system in PD are unclear, accumulating results have shown more and more PD risk genes are associated with autophagy and synaptic vesicle through human genetic studies and genomic analysis (Chang et al. 2017).

α-Syn has two forms of precursor: a naturally unfolded structure and a sacral helix which connect to the synaptic vesicle. Studies have shown that the α-helical structure of α-syn promotes the formation of the SNARE complex and regulates the process of endocytosis by binding to VAMP2 (Burré et al. 2010). Lacking of α-syn promotes the filling of synaptic vesicles and ultimately increases the secretion of DA. In addition to A30P mutation, studies have found that expressions of human wild-type α-syn or A53T and E46K mutant α-syn have developed extracellular secretory disorders of synaptic vesicles. Combined with the aforementioned α-syn function in autophagy, it is not difficult to see that α-syn is the main marker of PD, which also participates in the regulation of the synaptic vesicle cycle and the autophagy lysosomal system.

The *LRRK2* mutation is the most common type of mutation in familial PD (Zimprich et al. 2004). The LRRK2 is located in the synaptic compartment and interacts with endocytic-associated proteins to regulate the synaptic vesicle transport (Matta et al. 2012). *LRRK2* mutation slows down the endocytosis of synaptic vesicles (Zimprich et al. 2004), resulting in excessive activation of LRRK2 kinase, and disrupts normal degradation via autophagy. Recent studies have identified some substrates for LRRK2, including a range of Rab GTPases such as Rab8, Rab10 and Rab12 (Steger et al. 2016). Rab GTPases are involved in the regulation of numerous intracellular membrane trafficking. It has been reported that *LRRK2* mutation will directly lead to the changes in phosphorylation on Rab protein, which are likely to interfere with the construction of endometrial structures and invisible synaptic transmission and autophagic lysosomal transport.

EndophilinA is an important protein in the regulation of endocytosis by the clathrin in the nerve terminal, and it can bind to LRRK2 (Nalls et al. 2014). It has been found that LRRK2 can regulate the phosphorylation of EndoA, while the phosphorylated EndoA promotes the membrane deformation via a biased manner, and finally forms a

highly curved membrane structure to initiate the autophagosome formation (Soukup and Verstreken 2017). Therefore, mutations in *LRRK2* associated with PD can also interfere with the autophagy by affecting EndoA. On the other hand, the autophagosome EndoA was found to bind to the UPS E3 ligase FBXO32. The mutant mice without three types of EndoA subunits caused neurodegenerative change, significantly reduced LC3B, Atg5 and autophagosomes and increased FBXO3 levels. Therefore, researchers believe that disrupting EndoA or FBXO32 will cause autophagy or UPS system disorder, respectively, to increase the stress of residual degradation pathways. These changes will ultimately break intracellular protein homeostasis, and in some severe cases induce neurodegenerative lesions (Murdoch et al. 2016).

Synaptojanin1 is an inositol phosphatase enriched in synaptic precursors, which is often involved with auxilin in the clathrin-regulated synapse endocytic pathway. *SYNJ1/PARK20* and *DNAJC6/PARK19* are also considered as risk genes for early onset of atypical PD. Mutant mice lacking synj1 or auxilin exhibited abnormal accumulation of vesicles surrounded by clathrin at the synaptic sites. Similar to EndoA, synj1 can also be phosphorylated by *LRRK2* and involved in endocytosis. *SYNJ1* is also involved in the autophagy regulation. In the case of R258Q mutation in the synj1 SAC1 region, associated PD people found a disorder in lipid synthesis and caused WIPI2/Atg18a accumulation (Vanhauwaert et al. 2017). Little is known about the direct relationship between synj1, endocytosis and autophagy, which needs further research.

Dynamin and Rab GTPase are two proteins that belong to the GTPase family (Raimondi et al. 2011). Dynamin is a large GTPase responsible for membrane cleavage during synaptic vesicles. The Rab family, as a candidate protein regulating autophagy in neuron and synaptic vesicle endocytosis, belongs to the small molecule GTPase. Both dynamin and Rab GTPase have been reported to be regulated by LRRK2, in which dynamin binds to LRRK2, while Rab8, 10 and 12 in Rab GTPase are phosphorylated substrates of LRRK2 (Steger et al. 2016). In terms of autophagy regulation, dynamin2 regulates mTORC1 activation, but these related reports are still not enough. The mechanism by which the Rab family is involved in autophagy regulation is unclear, but numerous experiments have demonstrated a correlation between this family and autophagy regulation.

2.2.6 Neurotoxic Parkinson's Disease Model and Autophagy

PD animal models established by chemical poisons are widely used in research. These models have contributed greatly to the elucidation of the molecular pathogenesis of PD and the evaluation of anti-PD drugs. In the previous section, we have described the role of PD-related genes in the autophagy regulation and their effect during the PD course. In the following section, we will discuss the effects of PD toxins on autophagy in cell and animal models. Studies have shown that autophagy is significantly affected in the 6-OHDA, MPTP, rotenone, paraquat and other toxicants-induced PD models.

6-OHDA is the first poison used to selectively kill DA neurons in PD animal models. It can stably induce the DA neuron degeneration at the injection site and the dysfunction of motor nervous system. The main mechanism of 6-OHDA toxicity on DA neurons is to cause oxidative stress in cells. During this process, increased autophagosome, activated LC3 and an increase in the lysosome number were observed. The results indicated that autophagy acts as a protective mechanism that is activated early in the injury. However, sustained autophagy activation can over activate lysosomes, causing activation of lysosomal proteins such as cathepsin L, which can cause toxic damage to cells. 3-Methyladenine can attenuate 6-OHDA-induced striatal DA neuronal death by inhibiting autophagy, and the cyclin-dependent hormone inhibitor olomoucine inhibits the nuclear translocation of cathepsin L. and activate autophagy to protect cell from damage. In the latest study, it was found that 6-OHDA induces autophagy independent of mTOR, and autophagy in this model can assist the cell to secrete PARK7, which lack a secretary signal, into extracellular space (Urano et al. 2018).

MPTP can cause irreversible PD-like damage in humans and is widely used in mice to establish PD animal models. The lipid soluble MPTP is metabolized by MAO-B to MPP^+ after passing through the blood–brain barrier. MPP^+ enters into the DA neurons through the DA transporter and aggregates in the mitochondria, inhibiting the mitochondrial respiratory chain complex I function, and interfering with the ATP synthesis. At the same time, large numbers of free radicals are generated, causing DA neuron degeneration. In addition, MPTP can cause autophagosome accumulation, and lysosome reduction, while autophagy activator, rapamycin, can attenuate MPTP-induced DA neuron death, suggesting that autophagy inhibition is involved in MPTP toxicity. MPP^+ treated MN9D cells showed autophagosome and p62 accumulation, suggesting that autophagy degradation is inhibited. PC12 also showed autophagosome accumulation and aggregation of α-syn after MPP^+ treatment, which may be related to the inhibition of autophagosome degradation by MPP^+-induced kinesin inhibition. However, there are other reports that showed MPP^+-induced autophagic death in SK-N-SH and PC12 cells, which can be alleviated by autophagy inhibitors or knockout of autophagy-related genes. These different effects may be related to various factors such as cell type, drug concentration, treatment time and analytical methods (whether separating soluble and insoluble proteins and whether changes in autophagic flow are considered). MPP^+ may activate autophagy at an early stage, but the accumulation of ROS eventually inhibits autophagy because of the energy exhausting.

Long-term exposure to rotenone is a risk factor for PD. Rotenone has been widely used to establish PD animal models. The rotenone-induced PD model showed oxidative damage, α-syn aggregation, DA neuronal degeneration and pathological changes similar to human PD, such as α-syn-positive inclusion Lewy bodies. The mechanism involved in the rotenone-induced toxic damage is that rotenone can selectively inhibit mitochondrial oxidative respiratory chain complex I, resulting in mitochondrial dysfunction, oxidative stress and energy metabolism disorders. Rotenone treatment can cause autophagosome accumulation in SH-SY5Y neurons, an increased LC3-II, and p62 or α-syn aggregation. It is suggested that the lysosomal degradation pathway is

inhibited, which may be related to the reduction of cellular ATP levels by rotenone. Multiple autophagy-inducing agents such as rapamycin, lithium, valproic acid, carbamazepine and kaempferol can reduce the cytotoxicity of rotenone by up-regulating autophagy, indicating that autophagy inhibits the cell damage caused by rotenone.

Paraquat is very similar to the structure of MPP^+ and is widely used as a powerful insecticide. Paraquat can cause damage to the nigrostriatal pathway in animals, producing symptoms similar to PD. Its main toxic mechanism is also because of cell damage caused by the specific inhibition of mitochondrial oxidative respiratory chain complex I function, increase of ROS and reduction in ATP synthesis. Paraquat can increase the amount of α-syn in cells and lysosomes, accompanied by an increase in Hsc70 and LAMP-2A, suggesting that the CMA may be affected. Chronic paraquat treatment can increase protein aggregation in cells such as p62, and reduce the LC3-II formation and the autophagosome number. Rapamycin and other autophagy activators can alleviate paraquat-induced cell and animal damage and further support autophagy inhibition in paraquat toxicity. However, studies have shown that paraquat can activate autophagy in SH-SY5Y cells.

2.3 Autophagy Regulation in PD Treatment

In summary, autophagy is involved in the whole process of PD. Although inducing autophagy may occur at a certain stage or under the influence of specific toxicants, chronic autophagy inhibition is an important pathological change in PD from the perspective of the overall course of PD. This is also highly consistent with the PD features: accumulation of abnormal proteins. Inhibition of neuronal autophagy results in the reduced ability for cells to clear aging organelles, misfolded proteins and abnormal protein aggregates, which accelerates cell death. Therefore, people are looking for small molecules that can regulate autophagy and attempts to modulate autophagy-related genes to restore the autophagy function in neuron, and finally improved the neurodegenerative disease therapy such as PD.

2.3.1 Small Molecular Autophagy Regulators in PD Models

2.3.1.1 Rapamycin

Rapamycin, also known as sirolimus, is a clinically used drug for post-transplant immune rejection inhibition. In recent years, its application prospects in tumors, cardiovascular diseases and neurodegenerative disease have received great attention. Rapamycin target is the mammalian target of rapamycin (mTOR). mTOR is a serine/threonine protein kinase that plays a central role in cell growth, protein synthesis and autophagy regulation. Rapamycin first binds to the FK506 to form a complex that binds to the FRB domain of mTOR, interfering with the assembly of the mTOR

C1 complex to inhibit its kinase activity. Studies have shown that rapamycin not only prolongs the lifespan of a variety of biological species, including mice, but also shows significant protection against multiple neurodegenerative disease models.

In the MPTP-induced mouse PD model, intraperitoneal injection of rapamycin attenuated DA neuronal apoptosis in the nigrostriatal pathway, possibly by repairing autophagy and inhibiting the expression of the death-related protein RTP801. Rapamycin also attenuates motor dysfunction induced by 6-OHDA and Lactacysin intracerebral injection and nigrostain DA neuron degeneration, possibly by inhibiting mTORC1 activation and inducing autophagy. In the wild-type *SNCA* transgenic mouse model, intracerebral injection of rapamycin reduced aggregation of neuronal cell bodies and synaptic regions of α-syn, mainly by enhancing autophagy activity to activate soluble media functions. Feeding rapamycin also ameliorated the toxic effects of paraquat on *Drosophila* and improved the phenotype of *Parkin* or *PINK1* gene deletion in *Drosophila*.

2.3.1.2 Trehalose

Trehalose is a natural disaccharide that is widely distributed in many animals, plants and microorganisms in the world. Mammal does not synthesize trehalose, and people mainly intake it by eating fungi containing trehalose, seafood and plant products. Trehalose has been found to exhibit significant relief against a variety of neurodegenerative disease models such as PD, HD and ALS. Trehalose also showed good effects on a variety of PD models. It can eliminate the wild-type and mutant α-syn accumulated in neurons, alleviate MPTP-induced DA neuron damage in the substantia nigra–striatum region of mice, reduce α-syn, tau and other proteins accumulation caused by proteasome inhibition, and also inhibit the aggregation of α-syn in vitro by direct action. In view of the rich source of trehalose, cheap price and safety, it is a promising idea to apply trehalose to prevent and treat neurodegenerative disease. However, in some recent reports, the neuroprotective effects of trehalose and the effects on autophagy is controversial. Therefore, extensive researches need to do to fully confirm trehalose function.

2.3.1.3 Application of Lysosome-Targeting Modulators in PD Model

Lysosomes, which play the most direct degradation function in the autophagic lysosome pathway, have begun to be studied as a new target for PD treatment. By adding acidic nanoparticles to different PD models (*ATP13A2*, *GBA* mutant cells, MPTP-induced mouse model), the lysosomal pH can be significantly reduced, the lysosomal degradation function can be improved and apoptosis of DA neurons can be alleviated (Bourdenx et al. 2016). In addition, lysosomal-specific enzyme functions, such as glucosidase (GCase), can also be regulated. Studies have found that repairing glucoamylase expression by lentiviral infection would reduce α-syn accumulation and improves cognitive function in GD model mice (Sardi et al. 2013). Currently,

ambroxol, a drug partner used in respiratory diseases, has been demonstrated to repair Cathepsin D, Lamp2 and Saposin C activity. Now ambroxol has been used as a drug candidate for PD clinical trials.

2.3.1.4 Other Small Molecular Autophagy Regulators in PD Model

In recent years, new autophagy regulators have been discovered and applied to neurodegenerative disease research. The drug lithium agent for the treatment of bipolar disorder is an inositol monophosphatase inhibitor, which reduces intracellular inositol and Ins (1,4,5) P3 levels, and induces autophagy to promote α-syn degradation in neurons in PD through an mTOR-independent pathway. Two mood stabilizers, namely carbamazepine and sodium valproate, also reduce the toxic damage caused by rotenone by inducing autophagy. Some researchers have reported a non-mTOR-dependent autophagy activation loop, the cAMP/calcium/calmodulin loop. A variety of drugs acting on the loop such as clonidine, remidine, verapamil, calcium antagonists and calpain inhibitors can affect the autophagy level in cells and induce pathological aggregated proteins degradation. Recently, an alkaloid extracted from a natural product, guanidinamide, has been reported to restore the balance between autophagy and apoptosis levels by increasing BCL2 phosphorylation, attenuating the rotenone-induced PD models (Liu et al. 2018). Anti-diabetes drug, metformin, also plays a role in alleviating DAergic neuron degeneration, and in increasing striatal DA levels, and improving dyskinesia in MPTP-induced mouse PD models. In the meantime, metformin was also found to promote autophagy level and alleviate MPP^+-induced neuronal apoptosis in cell models. In addition, some other natural compounds isolated from plants including corynoxine B, corynoxine, resveratrol and polygala saponins have been reported to induce autophagy to promote the wild-type or mutant α-syn degradation.

It is a new strategy to find small molecular autophagy inducers in the nervous system for prevention of neurodegenerative diseases such as PD and has attracted great interest from researchers and clinicians. However, current researches are mainly performed on cell or animal models, and there is still a lack of support for large-scale clinical trial data.

2.3.2 Autophagy-Related Genes Regulation in PD Models

Autophagy is a highly conserved cellular mechanism with dozens of proteins involved in its regulatory pathway. Increasing or decreasing the expression level of certain autophagy regulatory genes in the nervous system by gene transfer can effectively and specifically regulate neuronal autophagy level. Animal experiments have shown that the regulation of the expression level of autophagy-related genes can alleviate the neurological phenotypes of animal models of PD.

BECN1 is the first identified mammalian autophagy-related gene that regulates autophagy activity by regulating the kinase activity of type III phosphatidylinositol 3-kinase (Class III PI3K). In one study, the researchers overexpressed the *SNCA* gene in B103 neurons through a lentiviral system, causing a series of changes, such as aggregation of α-syn in cells, decreased adhesion of neuron, impaired neurite outgrowth and tubulin skeleton alteration. At the same time, overexpression of the *BECN1* gene can reduce the α-syn level by about 50% and reverse the behavioral abnormalities caused by overexpression of *SNCA* gene (Zhu et al. 2014). The autophagy function and α-syn level changes caused by *BECN1* overexpression can be partially or completely blocked by the autophagy inhibitors 3-methyladenine and bafilomycin A1, and can be further enhanced by rapamycin (Zhu et al. 2014).

Transcription factor EB (*TFEB*) is a major transcription factor that regulates the autophagy–lysosomal system and up-regulates autophagy–lysosomal system function by initiating expression of series of autophagy-related genes and lysosomal synthesis genes. People overexpressed α-syn in the striatum region through the adeno-associated virus system induced by PD-related neurodegenerative changes such as decreased exercise capacity, DA-induced neuronal degeneration, decreased striatal DA content, and synapses dysfunction, accompanied by autophagic system function inhibition, lysosome number reduction and retention of TFEB in the cytosol. At the same time, overexpression of TFEB or *BECN1* gene significantly improved α-syn-induced PD-like damage, restored autophagy function and the lysosome number. In addition, changes in lysosome-related gene expression also play an important role in PD. Overexpression of LAMP2a in human SH-SY5Y cells, rat primary cortical neurons and *substantia nigra* DAergic neurons reduces α-syn accumulation and alleviates DAergic degradation.

2.4 Conclusion

The incidence of neurodegenerative diseases such as PD is getting higher and higher as the aging of Chinese population. At present, there are nearly 2.5 million patients with PD in China. The drugs used to treat PD are currently only for clinical symptoms, and there is no way to effectively alleviate the progressive course of the disease. In recent years, the important roles of autophagy in the pathogenesis of PD and other neurodegenerative diseases have been revealed by more and more studies. In fact, autophagy is involved in most of the pathogenesis mechanisms of Parkinson's disease. The protective effect of autophagy on cell and animal PD models also provides important evidence for autophagy as a new target for the PD treatment. Although the study of classical autophagy pathway has been well characterized, the autophagy regulation mechanism of the nervous system is still poorly understood. In addi-

tion, excessive autophagy induction can lead to cell damage, and how to regulate autophagy within a safe range is also a problem to be solved. The role of selective autophagy in pathological protein clearance has been widely reported. How to regulate selective autophagy and maintain the homeostasis of neuron in the process of pathological protein clearance is a question that needs further investigation. With the deepening of research on the pathogenesis of autophagy and PD, we expect that new drugs for PD can be developed by targeting autophagy, especially the selective autophagy, in the nervous system.

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Chapter 3
Autophagy and Motor Neuron Diseases

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Abstract Motor neuron diseases (MND) are a group of fatal progressive neurodegenerative diseases, which selectively affect the motor system in the anterior horn of spinal cord, brainstem, cortex and pyramidal tract. Motor neurons could be divided into two groups, which are upper groups in the motor cortex and lower groups in the brain stem and spinal cord. Loss of lower motor neurons leads to muscle weakness, wasting and cramps. Loss of upper motor neurons leads to brisk reflexes and functional limits. There are several types of motor neuron disease: amyotrophic lateral sclerosis (ALS), progressive bulbar palsy (PBP), progressive muscular atrophy (PMA), primary lateral sclerosis (PLS). Now, the studies of autophagy in MND focus on the type of ALS, so this chapter will summarize the alteration of autophagy in motor neurons, and how that knowledge contributes to our understanding of the pathogenesis of ALS.

Keywords Amyotrophic lateral sclerosis · SOD1 · TDP-43 · *C9orf72* · Lithium

3.1 Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by selective degeneration of motor neurons in the spinal cord as well as in brain stem. This disorder starts with focal weakness. Asymmetrical muscle weakness and muscle atrophy from the distal limbs are the most common clinical characters in ALS. The disease is globally distributed, with a prevalence rate of approximately

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(4–6)/100,000, an annual incidence rate of approximately (0.4–1.76)/100,000, and a mortality rate of 2/100,000.

ALS is the most frequent neurodegenerative disorder of midlife, with an onset in the middle-to-late 50s. About 10% of ALS cases are familial (fALS), while the 90% portion of ALS are sporadic with no family history (sALS). In sALS, the ratio of male to female patients may approach 2:1, while in fALS, the ratio is approximately 1:1. The time from the first symptom of ALS to diagnosis is approximately 12 months. As an abundance of ALS genes have now been identified, it will probably provide a better solution to realize the epidemiologic profile of ALS with stratification according to genetically defined subtypes. In 1993, Rosen and collaborators discovered that the gene encoding superoxide dismutase 1 (SOD1) has mutations in ALS patients. SOD1 is a ubiquitously expressed 153 amino acid protein involved in conversion of superoxide radicals to hydrogen peroxide. So far, more than 100 genetic variants throughout all coding regions have been associated with a risk of ALS, including point mutations, insertion and deletion mutations. SOD1 is a key enzyme involved in quenching toxic superoxide radicals within the cell. Mutations in SOD1 result in reduced stability of the enzyme leading to superoxide accumulation in cells, so SOD1 plays a crucial role in free radical degradation. Researchers have hypothesized that mutant SOD1 causes amounts of cellular defects such as mitochondrial dysfunctions, oxidative stress, calcium overload, abnormal protein aggregation, endoplasmic reticulum (ER) stress, axonal transport disruption, neurotransmitter misregulation, programmed cell death and inflammation. Among these cellular dysfunctions, abnormal protein aggregation is becoming the hot topic of this field. Accelerating the clearance of aggregated proteins through regulating autophagy is emerging as an attractive therapeutic strategy for ALS treatment.

With the advent of whole-exome sequencing, there has been a tremendous increase in discovery of new disease-causing genes in ALS, with ~30 different genes now implicated. The nuclear TAR DNA-binding protein 43 (TDP-43) has been identified as possibly causal gene for 4–5% cases of fALS. A hexanucleotide G4C2 repeat expansion in the chromosome 9 open reading frame 72 gene (C9orf72) is the most well-known genetic reason for ALS, which accounts for 30–40% of fALS, and it also results in frontotemporal dementia (FTD). Furthermore, a large number of genes have been linked to ALS, including fused in sarcoma (FUS), *sequestosome* 1(*SQSTM1*)/p62, optineurin (*OPTN*), TANK binding kinase 1(*TBK1*), VAMP-associated protein B (*VAPB*), valosin-containing protein (*VCP*), buiquilin2 (*UBQLN2*), charged multivesicular body protein 2B (*CHMP2B*), dynactin (*DCTN1*) and factor-induced gene 4 (*FIG4*). ALS genes discovery since 1990 is shown in Fig. 3.1 (Brown and Al-chalabi 2017).

Besides gene mutations, there are many related causes to induce ALS, including free radical oxidative damage, abnormal protein aggregation, excitatory amino acid toxicity, mitochondrial dysfunction, axonal transport disorder, apoptosis and immune defects. Like many other neurodegenerative disorders, an important pathological character of ALS is the mislocalization of proteins and presence of cytoplasmic aggregates in motor neurons, such as Lewy bodies, skein-like inclusions and Bunina inclusions, suggesting defects in the machinery that regulates protein homeostasis.

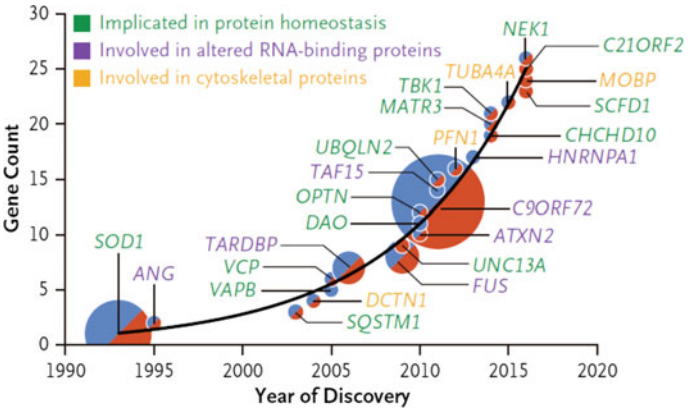

Fig. 3.1 ALS genes discovery since 1990

Cells have two major protein degradation pathways: the ubiquitin proteasome system (UPS) and the autophagy–lysosome pathway. The UPS, as the major proteolytic pathway in the cell, degrades short-lived, soluble proteins. Otherwise, autophagy is a sequential process, through which eukaryotic cells degrade long-lived proteins, misfolded proteins and impaired cytoplasmic organelles.

Although the pathogenesis of ALS is still unclear, pathological characteristics and genetic mutations associated with ALS have provided important clues on the etiology of ALS. Defects on the protein quality control systems (including autophagy) are closely related to age-dependent neurodegenerative diseases. Many genes associated with the pathogenesis of ALS are involved in the pathway of autophagy. In this chapter, we discuss the importance of the autophagy pathway in the context of ALS. In addition, we discuss the interaction of autophagy with other biological pathways relevant to motor neuron degeneration and provide perspective on the role of autophagy in the overall pathogenesis of ALS.

3.2 SOD1 and Autophagy

In 1993, the first genetic mutations were identified in the SOD1 gene in individuals with fALS. Further studies of fALS genomes have led to the discovery of more than 100 mutations in the SOD1 gene. Currently, SOD1 mutations account for approximately 12% of fALS cases and about 1% of sALS cases. SOD1 is a highly conserved cytoplasmic and mitochondrial antioxidizing enzyme that is involved in scavenging and converting toxic superoxide radicals into reactive oxygen species (ROS) and hydrogen peroxide. Although the mechanism by which mutant SOD1 (mSOD1) causes ALS is not fully understood, many studies suggest that the misfolding or aggregation of mSOD1 is a key event in the pathogenic process. Therefore, it will

provide a new direction for the effective treatment of ALS by exploring the mSOD1 degradation pathway in motor neurons.

3.2.1 Degradation of mSOD1 Protein

SOD1 protein is considered as one of the most important components of ALS-associated inclusions in motor neurons. Accumulating evidence indicates that a gain of toxic function of mSOD1 proteins is the cause of ALS. mSOD1 can be degraded by autophagy and by the UPS in neuronal and non-neuronal cells (Kabuta et al. 2006). The contribution of autophagy to mSOD1 clearance is comparable with that of the proteasome pathway. Further study reported that mSOD1 expression alone does not result in toxicity. Autophagy inhibition induced mSOD1-mediated cell death, suggesting that autophagy decreases the toxicity of mSOD1 proteins. Inhibition of autophagy also increased SOD1 levels in detergent-soluble and -insoluble fractions, indicating that both of them are degraded by autophagy.

3.2.2 SOD1 and Autophagic Flux

mSOD1 aggregation can affect multiple early steps in autophagy, with evidence from mSOD1 models pointing to the occurrence of hyperactive induction of autophagy. Upregulated LC3-II level and increased autophagic vacuoles are consistently found in spinal cord and brainstem fractions of SOD1G93A mice (Morimoto et al. 2007). Moreover, in spinal cords of SOD1G93A mice, there was persistent upregulation of the autophagy regulators TFEB and beclin1, with a corresponding decrease in mTOR level. However, autophagic flux describes the dynamic process of autophagy, which includes autophagosome formation, maturation, fusion with lysosomes, degradation and the release of macromolecules back into the cytosol.

Autophagosome maturation and subsequent lysosomal fusion are downstream events in autophagy, at which mSOD1 is also thought to exert its deleterious effects. Differentiated and highly polarized cells, such as neurons, exhibit spatially segregated pathways for autophagy. Autophagosomes are formed in the growth cone of neurons and at synaptic junctions and were rapidly transported to the soma via the microtubule-based motor cytoplasmic dynein and its activator dynactin. In mSOD1-expressing transgenic mice, it was found that there was colocalization between dynein and mSOD1. In mice, misfolded mSOD1 acquired a gain-of-interaction with dynein, defecting axonal transport and impairing autophagosome transport. Interestingly, overexpression of SNAPIN, a vesicle docking protein, can compete with mSOD1 for binding to dynein, thereby rescuing the effects of impaired retrograde transport via recruitment of dynein motors to late endosomes for transport (Xie et al. 2015).

Given the important role of autophagy in cleaning the misfolded proteins, promoting autophagy in mSOD1 models has been thought to be beneficial. However, the

assessment of autophagic enhancement in ALS SOD1G93A mice has yielded conflicting results. In 2008, it was found that autophagy inducer lithium carbonate could delay disease onset and prolong lifespan in SOD1G93A mice; however, the later study in 2009 reported that lithium carbonate worsens neuropathology in SOD1G93A mice. Furthermore, when SOD1G93A mice were treated with autophagy enhancers including trehalose and resveratrol, animals displayed delayed symptom onset, prolonged survival and diminished motor neuron loss. However, when SOD1G93A mice were treated with another autophagy enhancer, rapamycin, they showed accelerated motor neuron degeneration and shortened lifespan.

Parkin, an E3 ubiquitin ligase related to Parkinson's disease, allows the polyubiquitination of mSOD1, promoting their degradation by autophagy–lysosome system. The clearance of aggregated, mSOD1 by autophagy is mediated by Hsp 70 and its co-chaperone BAG3. Otherwise, the E3 ligase Mahogunin ring finger 1 (MGRN1) was shown to contribute to the clearance of toxic mSOD1 through autophagy. The reason behind the selective degeneration of motor neurons in SOD1-linked ALS is still not understood. One proposed idea argues that these motor neurons are less able to correctly degrade misfolded proteins. In addition, it appears that autophagy in motor neurons plays counteracting roles that vary depending on the phases of disease progression in the ALS transgenic mice. Using Atg7 conditional knockout SOD1G93A mice, autophagy in motor neuron is required to maintain neuromuscular innervation early in disease but eventually promote disease progression at an advanced disease state (Rudnick et al. 2017). More studies are required to directly manipulate autophagy in the context of ALS and define the possible impact in disease progression in mSOD1-associated ALS.

3.3 TDP-43 and Autophagy

TDP-43 was identified as a key factor of the ubiquitin-positive inclusions in both ALS and FTD patients. The difference between ALS and FTD in clinical and pathological features suggests a key role for TDP-43 in diverse disease pathogenesis. Mutations in TDP-43 contribute to 4–5% of fALS and nearly 1% of sALS cases, and most of the mutations are concentrated in the C terminus of the protein. Furthermore, TDP-43 immunopositive inclusions were found in the motor cortices and spinal cords of nearly 97% of fALS and sALS patients, suggesting an important consequence for the aggregation of the protein into inclusions. TDP-43 contains two RNA recognition motifs (RRM1 and RRM2) which bind to nucleic acids and a C-terminal glycine-rich domain that mediates protein–protein interactions. TDP-43 has important roles in RNA process and homeostasis, including transcription, splicing and transport of target mRNAs. Even though many advances have been made, how the gain- and loss-of-function mechanisms of TDP-43 contribute to ALS pathogenesis remains to be a complicated area of investigation.

3.3.1 Degradation of TDP-43 Protein

TDP-43 is accumulated in ubiquitinated inclusions in FTD with ubiquitin-positive inclusions and ALS diseased brains. TDP-43 forms pathogenic C-terminal fragments with low molecular weight as 25 and 35 kDa before aggregation. TDP-43 is localized in ubiquitin-positive compounds in patients' brains, which implies that UPS may be related with TDP-43 degradation or pathogenesis. Many studies have suggested that the protein levels of TDP-43 and TDP-25 were increased in cells treated with MG132, a proteasome inhibitor (Wang et al. 2010), or 3-MA, an autophagy inhibitor, but decreased by trehalose, an enhancer of autophagy. Furthermore, more protein level changes in TDP-25 than in TDP-43 were observed in cells treated with above-mentioned inhibitors or enhancer. Thus, these results demonstrate that both UPS and autophagy pathway contribute to the clearance of aberrant TDP-43 proteins, including TDP-25 and TDP-43.

Furthermore, TDP-43 accumulated in cytoplasmic aggregates, which are also positive for p62 and ubiquitin staining, in Hela cells depleted of *ESCRT-I* (*Tsg101*) or *ESCRT-III* (*Vps24*) (Maria et al. 2007). This result indicated that the degradation of TDP-43 is required for multivesicular bodies (MVBs) formation and autophagy pathway. Therefore, enhancing autophagy with drugs may be a tractable therapeutic strategy for TDP-43 associated diseases.

3.3.2 Loss-of-Function

TDP-43 is, for the most part, expressed in nucleus, where they carry out important functions in RNA metabolism in many ways, including transcriptional regulation, splicing, mRNA stabilization and miRNA processing. TDP-43 also regulates axonal transport and neuronal plasticity. In the case of ALS, TDP-43 is often observed in the cytoplasm, which corresponds to its mislocalization. Several studies support an important role for autophagy in TDP-43-linked ALS pathogenesis. TDP-43 depletion shows downregulation of ATG7 at both the mRNA and protein level, which is an integral constituent of the autophagosome formation. Furthermore, TDP-43 depletion also inhibited autophagosome–lysosome fusion by depleting dynactin, a motor protein essential for retrograde transport that enables vesicular fusion events in the pathway.

In addition, the loss of TDP-43 function in nucleus was reported to downregulate expression of mTOR, a negative regulator of autophagy. Recent study reported that the loss of TDP-43 strongly induced a nuclear translocation of TFEB (Xia et al. 2016), which is a regulator of lysosomal biogenesis and autophagy, by modulating the mTORC1 key component raptor. This regulation enhanced global gene expressions in the process of autophagy–lysosome biogenesis. However, loss of TDP-43

also destroyed the fusion between autophagosomes and lysosomes through dynactin 1 downregulation, resulting in aggregation of immature autophagic vesicles and overwhelmed autophagy–lysosome pathway function.

In summary, it is clear that the loss of TDP-43 function leads to a complex array of gene expression dysregulations that result in various aberrations to the events of autophagy; however, the combined effect of these changes on autophagic clearance remains uncertain.

3.4 C9orf72 and Autophagy

Ground-breaking progress was achieved by finding a hexanucleotide GGGGCC repeat expansion in the *C9orf72* gene, which is the most frequent genetic cause of diseases in both Europe and North America, but is extremely rare in Asia and the Middle East, indicating a different genetic architecture underlying ALS in these populations (Majounie et al. 2012). The majority (>95%) of healthy controls have $\leq$11 hexanucleotide repeats in the *C9orf72* gene. The pathological repeat-length threshold is still unclear; meanwhile, an arbitrary cut-off of 30 repeats is widely used, while larger expansions are most commonly observed in patients with C9-ALS ranging from hundreds to thousands of repeats. Three main mechanisms have been proposed: loss of function caused by a decreased expression of the C9orf72 protein, production of toxic *C9orf72* repeat RNAs, and accumulation of dipeptide repeat proteins produced by repeat-associated non-ATG translation (RAN).

3.4.1 Loss-of-Function of the C9orf72 Protein

Although little is known about C9orf72-coding protein function, an interesting recent study showed that the C9orf72 protein may function in endosomal trafficking and autophagy. Hexanucleotide repeat expansion within non-coding regions can cause loss of protein expression through failed translation of mRNA transcription. Haploinsufficiency has been suggested as a potential underlying mechanism for C9orf72-related ALS. Reduction in total and variant-specific *C9ORF72* mRNA has been reported in post-mortem tissue from C9-ALS patients (Belzil et al. 2013). To determine whether reduction or loss of C9orf72 might be related to disease, *C9ORF72* has been knocked down in zebrafish, mouse and human cell lines. However, these studies produced contradictory results, suggesting that C9orf72 protein may not be essential for normal nervous system function. Knockdown of *C9ORF72* in zebrafish resulted in significant shortening and abnormal branching of motor neuron axonal projections, and impaired behavior performance. However, knockdown of *C9ORF72* in mouse did not result in axonal defects or impaired phenotypic effects. Furthermore,

using antisense oligonucleotide to knockdown *C9ORF72* to ~10% of residual level had no significant phenotypic impact in C9-ALS patient-derived motor neurons. All these results suggest that loss of C9orf72 function may not be a key mechanism of the disease.

3.4.2 RNA-Mediated Toxicity

To date, most efforts to understand the mechanism underlying C9-ALS have exclusively focused on the contribution of RNA-mediated toxicity toward manifestation of the disease (Donnelly et al. 2013). The formation of RNA foci containing the repeat expansion is a key characteristic of many repeat expansion diseases associated with RNA-mediated toxicity. Both sense and antisense RNA foci of C9orf72 hexanucleotide repeats have been found in C9-ALS/FTD patient cells and tissues, and sequestration and dysregulation of RNA-binding proteins by hairpin and G-quadruplex structures consisting of GGGGCC repeats impair RNA processing and contribute to disease pathogenesis. However, it remains unclear whether these RNA-binding proteins associate with endogenous GGGGCC repeats or RNA foci in vivo.

3.4.3 Dipeptide Repeat Proteins Gain-of-Function Mechanism

Repeat-associated non-ATG (RAN) translation was initially reported to be associated with spinocerebellar ataxia type 8 (SCA8) and myotonic dystrophy type 1 (DM1), both of which involve a CAG repeat expansion. For the GGGGCC expansion in C9-ALS, RAN translation is carried out across three reading frames in the sense and antisense directions, generating six distinct poly-DPR proteins: poly-GA, poly-GP and poly-GR from the sense strand, and poly-PA, poly-GP and poly-PR from the antisense strand. RAN translation may extend beyond the repeat region such that five of the six reading frames can generate unique C-terminal fragments that could themselves influence stability or toxicity. Although DPR inclusions are widely distributed throughout the brain in C9-ALS patients, direct evidence on the role of RAN proteins in disease pathogenesis largely remains lacking. Each DPR has unique biophysical characters, which may determine its expression and aggregation pattern in brain. Further understanding of the ultrastructural features of DPR aggregates could shed light on toxicity mechanisms. Studies report that most poly-GP is soluble, whereas the majority of poly-GA is insoluble. Poly-GA inclusions colocalize RNA foci, leading to abnormal protein aggregation in motor neurons. Moreover, poly-GR and poly-PR, which coaggregate with several ribosomal proteins, were identified to be cytotoxic, most likely by interfering with RNA biogenesis in nucleoli, suggesting

that RAN proteins may play important roles in disease pathogenesis. However, different DPR inclusions co-occur and interact within individual neurons has yet to be studied systematically in C9-ALS patients.

3.4.4 C9orf72 and Autophagy

C9orf72 containing RNA foci are observed in 25% of the spinal cord and frontal cortical neurons of patients with repeat expansion. In C9-ALS patients, cytoplasmic inclusions are also positive for p62 in the spinal cord and brain tissues. Levels of p62 and LC3 are also increased in mice lacking C9ORF72. Recent study demonstrated that C9orf72 is a full-length distant homologue of proteins related to DENN, a guanine exchange factor (GEF) for Rab GTPases. Rab GTPases regulate membrane trafficking, and C9orf72 may be extensively involved at different process of autophagy by affecting vesicular trafficking. Furthermore, the role of C9orf72 in Rab-mediated process in the autophagy–lysosomal pathway was supported by the observation that physical interactions between C9orf72 and Rab7 and Rab11 GTPases are involved in late endosome maturation or endosome recycling, respectively, and a role of C9orf72 in autophagy regulation (Farg et al. 2014). In addition, one recent study suggested C9orf72 could modulate filamentous actin (F-actin) assembly (Sivadasan et al. 2016). F-actin is considered to play central role in the structure and dynamics of the cytoskeleton, which might be critical in the autophagy–lysosomal pathway. These results indicated the possibility of another mechanism through which C9orf72 may affect autophagy.

Otherwise, depletion of C9orf72 protein appears to inhibit autophagy initiation via the ULK1-mediated pathway. First, C9orf72 controls the Rab1a-dependent trafficking of ULK1 autophagy initiation complex. Under starvation condition, C9orf72 was found to localize on the surface of lysosomes in cells expressing endogenous tagged C9orf72 via CRISPR-Cas9 system. Secondly, C9orf72 may be essential for relaying signals from the ULK1 complex to promote downstream Rab-mediated phagophore maturation. Loss of C9orf72 function was reported to cause a defect in mTOR signaling pathway. In the absence of C9orf72, mTOR, a negative regulator of autophagy, was inactivated. It was observed that mTOR inactivation leads to the nuclear translocation of transcription factor TFEB, a master inducer of autophagy and lysosome biogenesis genes, resulting in an increase in autophagic flux.

Surprisingly, recent studies have indicated the implication of C9orf72 in a protein complex containing SMCR8 (another DENN domain-containing protein) and WDR41 (autophagy–lysosome pathway regulator). These proteins have also been found on the membrane of lysosomes. Altogether, current findings support a defect of both UPS and autophagy pathway in C9-ALS patients. The function of C9orf72 in the autophagy–lysosome pathways needs to be further studied.

3.5 Other ALS-Related Genes and Autophagy

3.5.1 Ubiquilin-2

Ubiquilin-2 is a 624-amino acid multidomain adaptor protein critical for maintaining protein homeostasis. In 2011, mutations in ubiquilin-2 have been shown to cause dominant x-linked inheritance of ALS and ALS/dementia (Deng et al. 2011). To date, more than ten mutations in the *UBQLN2* gene have been found in ALS or ALS-FTD patients. Moreover, either fALS or sALS patients have wild-type ubiquilin-2, which links ubiquilin-2 to the pathogenesis of ALS. *UBQLN2* gene encodes for the ubiquilin-2 protein containing an N-terminal ubiquitin-like domain (UBL) and a C-terminal ubiquitin-associated domain (UBA). The UBA domain binds poly-ubiquitinated proteins, while the UBL domain binds the cap of the proteasome. Thus, ubiquilin-2 delivers poly-ubiquitinated proteins to the proteasome for degradation. Furthermore, ubiquilin-2 is also involved in autophagy by interacting with LC3, suggesting that it participates in delivering cargo to autophagosomes. Transgenic rats expressing $UBQLN2^{P497H}$ recapitulated signs of UBQLN2-related ALS, indicating a toxic gain-of-function in motor neurons (Wu et al. 2015). In contrast, another study showed that mice expressing UBQLN2 mutants develop motor neuron disease, but mice overexpressing wild-type UBQLN2 were mostly devoid of clinical and pathological characters of ALS. Furthermore, recent evidence suggests that UBQLN2 interacts with OPTN at endosomal vesicles and acts in concert to facilitate endosome trafficking during autophagy. Considering its dual role in UPS and autophagy, it is yet to be determined if the ALS-associated mutations in UBQLN2 or OPTN affect protein degradation predominantly through one, or both, pathways. Further investigations could shed extensive information on this issue.

3.5.2 VCP

Valosin-containing protein (VCP) was originally discovered in an Italian family whose R191Q mutation resulted in autosomal dominant ALS. VCP is an essential AAA+ -ATPase involved in a range of cellular processes that include DNA replication and repair, cell cycle regulation and protein clearance. Loss of VCP activity results in autophagosome accumulation. After autophagy induction, autophagosomes fail to mature into autolysosomes in VCP mutant models. There are several proposed mechanisms by which VCP interacts with the autophagic machinery. With regard to autophagic initiation, VCP expression repressed the negative autophagy regulators mTOR, by upregulating GSK-3β, a positive autophagic regulator (Yeo et al. 2016). Other evidence suggests that mutant VCP interferes with the processes of autophagosome–lysosome fusion, since cells expressing VCP mutations exhibit accumulations of irregular, enlarged autophagic vacuoles, implying that autophagic flux defect. Extensive evidence suggests that the roles of VCP in autophagy are varied, and a

large number of studies are needed to further investigate the role of VCP in autophagy regulation and its role in the pathogenesis of ALS.

3.5.3 Sequestosome 1/P62

p62, also known as SQSTM1, is an adaptor protein involved in multiple cellular activities. Mutations in the p62 gene have been found to cause both ALS and FTD. p62 has been involved in the ubiquitin–proteasome system (UPS) and autophagy. Protein aggregates containing p62 have been found in many disorders, including ALS and FTD.

It is worth mentioning that mutations in p62 also cause Paget's disease of bone (PDB), a skeletal disorder, with the majority of PDB-associated p62 mutations residing in the UBA domain. Similarly, p62 missense mutations of UBA were also identified in patients with ALS-FTD. However, it remains unclear whether these shared mutations affect autophagy in both diseases. Limited evidence indicates that disease-associated mutations may confer loss-of-function of p62 in autophagy. For example, expression of the p62 P392L mutant, an ALS-associated UBA domain mutation, impaired basal autophagy in murine osteoclasts, implicating this mutation to impeded autophagic clearance. However, knockdown of p62 in zebrafish causes a locomotor phenotype that is rescued by expression of wild-type p62, but not P392L mutant p62. Furthermore, ALS-associated mutation p62 LIR mutant, L341V, is defective in recognition of LC3-II, hindering its functional involvement with autophagic vacuoles. A recent study suggests that p62 regulates the levels and functions of other ALS-associated proteins such as SOD1 and TDP-43, which is consistent with an important role of the misregulation of p62 in the dysfunction of protein homeostasis in the disease process of ALS.

In addition to canonical interactions between p62 and ubiquitinated inclusions, p62 selectively binds mutant SOD1 to form aggregates in the animal model of ALS. Early Previous study reported that accumulation of p62 correlates with an increase in aggregation of mutant (G93A-SOD1), but not wild-type SOD1 (Gal et al. 2007). This association was reported to be independent of the UBA domain, instead interacting via a distinct SOD1 mutant interacting region (SMIR) to enable sequestration of mSOD1. In the mouse models, knockdown of p62 was reported to shorten the lifespan of SOD1H46R and SOD1G93A mice of ALS. In the SOD1H46R mouse model, loss of p62 aggravated ALS symptoms, provoking accelerated weight loss, motor dysfunction and MN degeneration.

3.5.4 Optineurin (OPTN)

Genetic analyses have uncovered OPTN mutations in cohorts of ALS patients, which account for approximately 3% of fALS and about 1% of sALS. OPTN is a cytoplasmic protein containing 577 amino acids, involved in a diverse range of cellular processes, including vesicle trafficking, autophagy and NF-κB signal transduction. OPTN contains a ubiquitin-binding domain (UBN) in the C-terminal, linking to ubiquitinated substrate proteins; it also contains an LC3-related domain (LIR) that binds to LC3 and relates to autophagy (Korac et al. 2013). OPTN acts as an autophagy receptor whose role in degradation of damaged mitochondria has come to prominence. An important role for OPTN has been demonstrated in mitophagy, which is the selective degradation of damaged mitochondria by autophagy. In addition to playing a role in mitophagy, OPTN might also regulate autophagic flux. Therefore, the roles of OPTN in autophagy are diverse; it acts as not only a recognition receptor of substrate proteins but also a modulator for autophagy. More studies were needed to confirm the relationship between OPTN and autophagy in ALS.

3.5.5 CHMP2B

Charged multivesicular protein 2B (CHMP2B) is a subunit of the endosomal sorting complex required for transport (ESCRT-III). ESCRT-III is essential for the formation of MVBs and for the sorting of proteins at the endosomal membrane. Mutations in ESCRT-III block endosomal maturation lead to abnormal aggregation of autophagosomes. CHMP2B mutations (T104N, I29V, Q206H) have been found in ALS cohorts, with patients' brains exhibiting immature autophagosome accumulation and upregulated LC3-II and p62 levels, implicating autophagic flux impairment (Skibinski et al. 2005).

In cells, $CHMP2B^{Intron5}$ overexpression inhibited autophagic clearance, inducing the formation of ubiquitin and p62-positive inclusions. In addition, $CHMP2B^{Intron5}$ displays impaired endosomal recruitment of Rab7, a GTPase required for endosome–lysosome fusion. Experiments in a Drosophila model indicated that $CHMP2B^{Intron5}$ inhibits autophagosome maturation by repressing the function of an endosomal SNARE, which was involved in the maturation of phagophores into closed autophagosomes (Urwin et al. 2010). Furthermore, C-terminal CHMP2B truncations appear to exert deleterious effects that disrupt various steps in autophagy, primarily at the late-stage endosome–lysosome fusion.

3.5.6 TANK-binding enzyme 1 (TBK1)

In 2015, by sequencing the whole exon in 2869 ALS patients and 6405 age–sex-matched controls, Cirulli et al. found that TANK-binding enzyme 1 (TBK1) gene was closely associated with ALS. TBK1 mutations have been found to be a rare cause of ALS in Chinese populations. Non-sense and frameshift mutations cause major disruption to TBK1 and may decrease its expression, implying that TBK1 haploinsufficiency contributes to the development of ALS. TBK1 belongs to the IKK-kinase family of kinases that are involved in innate immunity signaling pathways. TBK1 also has a major role in autophagy and mitophagy, operating the phosphorylation of autophagy adaptors, such as p62 and OPTN. TBK1 phosphorylation of the UBA domain of OPTN on Ser-177 enhances LC3 binding affinity and promotes autophagosome formation. It has also been reported that TBK1 coordinates with the autophagic machinery via phosphorylating Ser-403 on the UBA domain of p62, enhancing the affinity of UBA for K48/K63-linked ubiquitinated proteins (Pilli et al. 2012). Furthermore, TBK1 has also been implicated in autophagosome maturation. Maturation of autophagosome into autophagolysosomes was inhibited in TBK1 knockdown cells. Further study found that TBK1 regulates microtubule dynamics and the cytoplasmic levels of dynein, indicating that loss of TBK1 causes ALS by the impaired maturation of autophagolysosomes. In addition, several studies found TDP-43 and p62 positive inclusions in the motor neurons of various brain regions of TBK1 mutated ALS/FTD patients. These findings, particularly of p62 inclusions, provide further indications that TBK1 mutations may contribute to ALS through impairing autophagy process.

3.5.7 Factor-Induced Gene 4 (FIG4)

Factor-induced gene 4 (FIG4) encodes the protein FIG4. Mutations in FIG4 have been identified in 1–3% of ALS patients in Europe. These mutations are often deleterious, causing loss-of-function of the protein. FIG4 regulates the cellular level of PI(3,5)P2 maintaining endomembrane homeostasis and endosomal trafficking. PI(3,5)P2 is recognized by lysosomes and merges to gain access to the lysosomal machinery. Loss of FIG4 function in mutant mice shows motor neuron degeneration and enlarged endosomal vesicles, suggesting inhibition in the endo-lysosomal pathway. The effect of FIG4 depletion on autophagic clearance has been further substantiated in a mouse model, in which p62/SQSTM1 and LC3-II were observed to accumulate in neurons and astrocytes of FIG4-knockout mice. A defect in autolysosome clearance was also suggested by an abnormal accumulation of LAMP-2-positive vesicles in cultured FIG4-knockout neurons and astrocytes (Ferguson et al. 2009). Together with other endosome- associated proteins such as CHMP2B and alsin, the linkage of FIG4 to ALS further underscores the importance of endocytosis and autophagy in neurodegeneration.

3.6 Dysfunction of Autophagy in ALS

A crucial pathological feature of ALS includes accumulation of insoluble protein aggregates in degenerating motor neurons in the spinal cord, hippocampus, cerebellum and frontal and temporal cortices. Formation of misfolded protein aggregates is a normal physiological phenomenon. So, activation of autophagy may enhance the removal of toxic protein aggregates which protect some neurodegenerative diseases. However, emerging evidence supports the point of view that dysfunction of autophagy contributes to neurodegeneration. Some studies reported that overactive or dysfunctional autophagy may induce neuronal cell death in certain diseases. It is important to note that impairment of autophagosome degradation may cause physiological dysfunction of neurons. In this section, we discuss the contribution of autophagy dysfunction in SOD1-G93A mice models of ALS. Furthermore, we examine the crosstalk between autophagy and the important genes implicated in ALS pathogenesis.

3.6.1 Altered Autophagy in SOD1-G93A Mice

Mice-expressing mutant SOD1 shows an accumulation of aggregated proteins (including mutant SOD1) in the spinal cord, which is comparable with findings in human ALS, and has been widely used to model ALS. Accumulating evidence demonstrates that genetic mutations of SOD1 induce ALS through a dominant toxic gain-of-function rather than the loss of enzymatic function. Studies report the altered autophagy in G93A mice that starts from the presymptomatic stage of the disease (Li et al. 2008). It has been reported that inhibition of motor neuron autophagy in SOD1-G93A mice induces neuromuscular denervation in the early stages of the disease. However, it is not known whether the increased autophagic vacuoles in motor neurons is the result of autophagy induction or autophagy flux impairment. In other studies, hyperactivity of the autophagy pathway was detected in motor neurons of SOD1-linked ALS mice, which may account for the accumulation of autophagosomes in transgenic mice-expressing mutant SOD1-G93A. It is unclear whether the activation of autophagy or impairment of autophagy flux may increase autophagic vacuoles in motor neurons. Accumulating evidence suggests that defects in autophagic flux or in specific autophagy-regulatory processes, rather than simple induction, may contribute to the motor neuron degeneration. A recent report indicates that mTOR signaling pathway was significantly inhibited in G93A mice treated with rapamycin, inducing accumulation of autophagic vacuoles, but fails to decrease aggregation of mutant SOD1 in the spinal cord of transgenic G93A mice. This study indicates that autophagy may be disrupted in ALS. However, it is intriguing to propose that the disturbance of autophagy pathway contributes to the toxicity induced by mutant SOD1, and modulation of autophagy may be a potential strategy for SOD1-mediated ALS treatment.

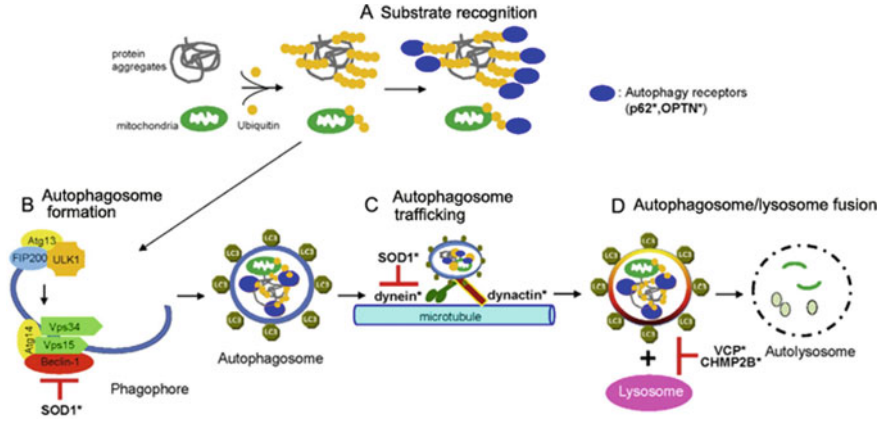

Fig. 3.2 Autophagy defects due to ALS-associated gene mutations

3.6.2 Defects at Different Stages of Autophagy in ALS

Figure 3.2 shows the ALS-associated gene mutations-mediated autophagic defects (Lee et al. 2015).

3.6.3 Initiation Stage: Substrates Sequestration

It is now well accepted that selective autophagy plays a major role in the clearance of various cellular structures, including protein aggregates and abnormal mitochondria. The clearance of protein aggregates requires the ubiquitination of autophagic substrates. Molecules that bind both polyubiquitinated protein and membrane components of the autophagosome mediate the selective autophagic process. p62 is an autophagy receptor that plays a major role in the clearance of protein aggregates. Substrate recognition by p62 is not limited to misfolded protein aggregates, but also includes organelles such as mitochondria. It is envisaged that mutations in ubiquilin-2 may change the ubiquitin modification on substrates, influence recognition by p62 and reduce the clearance of ubiquitinated misfolded proteins. Otherwise, OPTN, another autophagy receptor, which barely has ALS-associated mutations, interferes with autophagy-induced degradation of abnormal proteins. The TBK1-mediated phosphorylation of the autophagy receptors, such as p62 and OPTN, may modulate the recognition of autophagic cargoes by the receptors, causing autophagic flux impairment. Therefore, whether TBK1 plays a role or not in the pathogenesis of ALS needs further research.

3.6.4 Maturation Stage: Autophagosome–Lysosome Fusion

In SOD1-G93A mice, rapamycin treatment increases autophagic vacuoles in the motor neurons of spinal cord. However, it causes accumulation of p62, a marker of autophagic flux, which is degraded by autophagy. The increased autophagic vacuoles and accumulated p62 raise the possibility of impaired autophagosomes clearance in SOD1-G93A transgenic mice, which may result from the inhibition of the fusion between autophagosomes and lysosomes and/or impaired protein degradation in the autolysosome. As a membrane trafficking pathway, autophagy carries cytoplasmic components to the lysosome for degradation. During this process, autophagosomes move to the lysosome in a microtubule and dynein/dynactin complex-dependent manner. Although microtubules are dispensable for autophagy, they are essential for the fusion step in mammals. Mutations in dynein or dynactin affecting the dynein machinery cause defective autophagosome movement and autophagosome–lysosome fusion in the motor neurons. It is speculated that mutant SOD1 protein may impair autophagosomes clearance through inhibition of dynein/dynactin function in SOD1-G93A transgenic mice. Similar autophagosome–lysosome fusion defects have also been observed in the mouse models of CHMP2B or VCP mutations. Mutations in CHMP2B disturb endosomal maturation and lead to abnormal aggregation of immature autophagosome and upregulated level of p62, suggesting the impairment of autophagosome–lysosome fusion. Moreover, VCP mutation leads to an accumulation of autophagosomes, which fail to degrade aggregated proteins due to impaired autophagosome–lysosome fusion. These observations confirm the autophagic dysregulation in ALS, with potential impact on developing effective therapies.

3.7 Therapeutic Effects of Autophagic Inducers on ALS

Amyotrophic lateral sclerosis remains a fatal neurodegenerative disease destroying the primary motor system. Many sporadic cases were possibly caused by some identifiable genetic causes, which are potential drug targets. Nevertheless, efforts in elucidating the pathogenesis of ALS have achieved significant advances. With accumulating research evidences of these pathologic mechanisms, we expect that environmental factors may play an important role in it. Riluzole and the recently approved edaravone have shown good therapeutic effect on ALS, which however still needs more treatments. Neurotrophic factors, vascular endothelial growth factor, anti-apoptosis, anti-inflammatory drugs and stem cell transplantation have shown neuroprotective effects in the transgenic animal models. The reports that defective autophagy may contribute to the pathogenesis of neurodegenerative diseases suggest that activation of autophagy may exhibit potential therapeutic effect.

3.7.1 Lithium

Lithium carbonate is a current first-line medicine for treatment of bipolar disorder and depression, which exhibits neuroprotective effects in several types of diseases models, such as cerebral ischemia and frontotemporal lobe epilepsy. Lithium induces autophagy by inhibiting the IMPase, which leads to reduced levels of free IP3 and myo-inositol-1,4,5-triphosphate cellular levels. Since IP3 has been shown to suppress autophagy, depletion of IP3 by lithium leads to the activation of autophagy. Thus, the effect of lithium against glutamate probably depends on its ability to deplete the IP3 from the cell. In other words, lithium and glutamate produce a contrary regulation effect, respectively, of the autophagy pathway. Lithium targets different molecules, affecting multiple pathways, which depends vitally on the dose. Low drug doses inhibit IMPase (Ki 0.8 mM) activity, thereby inducing autophagy, while high drug doses inhibit the activity of glycogen synthase kinase (GSK-3β) (Ki 2 mM), leading to negative regulation of autophagy. Moreover, lithium has been shown to act on other pathways as well, involving extracellular signal-regulated kinase (ERK), or PI3k/Akt. All the pathways have an impact on the regulation of autophagy.

Based on these studies, Fornai et al. began to explore the effects of lithium carbonate on the pathogenesis and survival of SOD1-G93A mice since 2008 (Fornai et al. 2008a, b). They found a marked neuroprotection by lithium, which delayed the disease onset and duration and augmented the lifespan in SOD1-G93A mice. These effects were accompanied by the activation of autophagy and increased mitochondria in motor neurons together with suppressed reactive astrogliosis. Again, lithium increased the survival of motor neurons and reduced the slow necrosis which is characterized by mitochondrial vacuolization and that were severely affected in saline-treated G93A mice. Then, they conducted a 15-month parallel-group randomized study of adults with patients diagnosed with ALS. The clinical study was performed on 44 patients. No familial case was present. Sixteen patients were randomly divided into groups which receive riluzole (Rilutek 50 mg, 1 tablet × 2/day) plus lithium (Carbolithium; two daily 150 mg doses of lithium carbonate), and the remaining 28 patients received riluzole only. Patients treated with lithium didn't die during the 15 months of the follow-up. Furthermore, compared with control patients treated with riluzole for the same amount of time, patients treated with lithium were markedly attenuated in disease progression. These results suggested that lithium carbonate can significantly delay the disease progression of ALS, and improve the movement capacity and neuromuscular function in ALS patients.

Furthermore, lithium and valproic acid (VPA) are two primary drugs used to treat bipolar disorder (Feng et al. 2008). Both of them have neuroprotective effects in vivo and in vitro. Drug combination of lithium and VPA was reported to have synergistic neuroprotective effects against glutamate excitotoxicity in cultured brain neurons, as well as inhibiting glycogen synthase kinase-3 (GSK-3) activity through enhanced GSK-3 phosphorylation. The 30-day-old SOD-G93A ALS mice were injected twice a day intraperitoneally with LiCl (60 mg/kg), VPA (300 mg/kg) or lithium plus VPA until death. The results indicate that compared with the results of monotreatment with

lithium or VPA, the drug combination group has better and more consistent effect in delaying the onset of disease symptoms, prolonging the survival time and decreasing the neurological deficit scores. In addition, clinical trial also showed that lithium and valproate cotreatment significantly increased survival, and exerted neuroprotection in ALS patients.

However, compared to vehicle-treated mice, Pizzasegola et al. reported a significant anticipation of the onset and reduced survival in 129 Sv/G93A and no effect in C57/G93A female mice treated with lithium (Pizzasegola et al. 2009). Furthermore, mice treated with lithium neither exerted neuroprotective effects nor increased protein level of LC3 and the activity of mitochondrial complex IV in the spinal cord, which imply no therapeutic or neuroprotective effect of lithium on SOD1-G93A female mice. At the same time, using SOD1-G93A mice and ALS human patients, including sibling matching, gender balancing, investigator blinding, and transgene copy number verification for each experimental subject, studies minimized the likelihood of attaining a false positive therapeutic effect in this standard animal model of familial ALS (Gill et al. 2009). Results from this study do not support taking lithium carbonate into human clinical trials for ALS. These studies indicate that more autophagic regulators should be used to test the therapeutic effects of autophagy in ALS.

3.7.2 Other Autophagic Inducers

Potential therapeutic effects of autophagy inducers in ALS are shown in Table 3.1.

3.7.2.1 Rapamycin

mTOR is a 289 kDa, serine/threonine protein kinase, which is sensitive to growth factors, amino acids and energy status of the cell. mTOR and its protein complexes offer exciting and unique avenues of intervention in ALS through the oversight of programmed cell death pathways of apoptosis and autophagy. Clinical strategies for ALS that implement mTOR must achieve parallel objectives to protect neuronal survival, considering the ability of mTOR to broadly impact cellular function. Rapamycin is a widely used autophagy enhancer, which induces autophagy by inhibiting mTOR. Rapamycin binds to its intracellular receptor FK506, and subsequently this complex binds to mTOR. After this ternary complex is formed, the kinase activity of mTOR is attenuated. Otherwise, CCI-779, or temsirolimus, is an ester derivative of rapamycin. Comparing with rapamycin, CCI-779 is more water soluble and a prodrug metabolized to rapamycin in the body.

In 2011, Le's lab first reported the effects of rapamycin treatment on the disease course in the SOD1-G93A animal model of ALS (Zhang et al. 2011). This study indicated that autophagic alteration occurs primarily and specifically in the motor neurons of spinal cords of the ALS mice at a relative early stage of the disease. Impairment of autophagic pathway may contribute to the selective motor neurons

Table 3.1 Potential therapeutic effects of autophagy inducers in ALS

Mode of action	Autophagic inducer	Therapeutic effects
mTORC1 inhibition	Rapamycin	Attenuates ALS-like features and TDP-43 AGGREGATION IN tdp-43 transgenic mice Reduces accumulation of FUS-positive stress granules and neurodegeneration induced by ALS-linked ALS mutation Has no beneficial effect in mutant SOD1 transgenic mice Has beneficial effects in mutant SOD1 transgenic mice lacking mature lymphocytes Augments disease progression in mutant VCP transgenic mice Attenuates ALS-like features and TDP-43 aggregation in TDP-43 transgenic mice
AMPK activation	Lithium	Has beneficial effects in mutant SOD1 transgenic mice Has no effect in mutant SOD1 transgenic mice
Unknown	Trehalose Spermidine Carbamazepine	Ameliorates ALS-like features in mutant SOD1 transgenic mice Attenuates ALS-like features and TDP-43 aggregation in TDP-43 transgenic mice Ameliorates ALS-like features in mutant SOD1 transgenic mice

degeneration in ALS, and rapamycin is found to accelerate the disease course and neuropathological processes in the ALS mice through the activation of the apoptotic pathway and other mechanisms. Another study reported that rapamycin did not extend disease onset and survival in the SOD1-H46R/H48Q mouse model of ALS, which indicate that the pathogenic mechanisms in G93A and H46R/H48Q mice are distinct (Bhattacharya et al. 2012).

Mutations in VCP cause ALS and FTD, which is necessary for protein degradation via lysosome and proteasome. Using VCP-R155H mutant mouse model of ALS, Ching et al. found mutations in VCP disrupt autophagosome and endosome maturation resulting in vacuolation, weakness and muscle atrophy (Ching and Weihl 2013). The phosphorylation of mTOR targets was decreased and autophagosome biogenesis was increased in VCP-R155H muscle. Furthermore, rapamycin aggravated symptoms of weakness, atrophy and vacuolation in VCP-R155H mice, which was accompanied by the aggregation of autophagic substrates such as p62, LC3II and ubiquitinated proteins. The above results indicate that mTOR signaling pathway may be disrupted by VCP mutations, which contributes to ALS disease pathogenesis. Treatment with rapamycin, the mTOR inhibitor, on some autophagic disorders may worsen disease.

3.7.2.2 Trehalose

Autophagy is regulated by mTOR-dependent and mTOR-independent pathways that are amenable to chemical perturbations. Various mTOR-independent autophagy pathways have been described, besides the regulation of autophagy by mTORC1 and diverse upstream signals impinging on it. The first mTOR-independent regulation of autophagy defines a role for the inositol signaling pathway that negatively regulates this process. In addition, autophagy is also regulated by changes in intracellular Ca^{2+} levels and the second messenger, cAMP.

Trehalose is a non-reducing disaccharide, which is present in many organisms, including bacteria, yeast, insects, fungi and plants, except in mammalian cells. By preventing denaturation and aggregation of proteins through direct protein–trehalose interactions, trehalose acts as a potent autophagy activator. In 2014, Le's lab reports that trehalose can delay disease onset and prolong the lifespan in the SOD1-G93A mouse model of ALS (Zhang et al. 2014). Pathologically, trehalose not only protects the motor neurons from degeneration in the anterior horn of spinal cord, but also attenuates muscle atrophy by restoring muscle functions and inhibiting oxidative stress. This effect may be a potential neuroprotective mechanism of trehalose on motor neurons in the ALS model since emerging evidence has shown that skeletal muscle might be a primary site of disease to initiate non-autonomous motor neuron degeneration in ALS.

At the same time, Castillo et al. reported that administration of trehalose to SOD1-G86R transgenic mice significantly prolonged lifespan and attenuated the progression of disease signs (Castillo et al. 2013). Decreased accumulation of SOD1 aggregates and enhanced motoneuron survival were associated with these effects. Consistent with the above results, treatment with trehalose enhanced the nuclear translocation of FOXO1, an important transcription factor involved in autophagy activation in neurons, which supports further approaches by treatments with trehalose and enhancers of mTOR-independent autophagy for the treatment of ALS.

Furthermore, an investigating test with 50,000 compounds to find novel autophagy modulators showed various small-molecule enhancers (SMERs) and small-molecule inhibitors (SMIRs) of the cytostatic effects of rapamycin in yeast, and further screening identified several autophagy-inducing SMERs (SMER10, SMER18 and SMER28) and autophagy-inhibitory SMIRs in mammalian cells. Many structural analogs of these SMERs induce mTOR-independent autophagy. More studies are needed to support the effects of the novel autophagy modulators in ALS.

3.8 Conclusion

Many effective therapeutic interventions in in vivo studies have failed to achieve success in clinical applications for patients with ALS. And yet for all that, studies and approaches have continuously been established to define the molecular pathogenesis of these devastating diseases. Development of more specific autophagy modulators

is an urgent need, considering the therapeutic potential of autophagy upregulation in neurodegenerative disease. However, emerging evidence supports the point of view that impaired autophagic flux contributes to the neurodegeneration in ALS. Several important genes implicated in ALS pathogenesis are involved in the autophagic dysfunction in different stages. Manipulating the autophagy process is thus a complicated dilemma. It is anticipated that more specific autophagic regulators will be discovered and deeper understanding of the autophagy biology will be advanced in the near future, which will help decode the mystery of autophagy in ALS pathogenesis, and evaluate the therapeutic value of autophagy modulators for this devastating disease.

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Chapter 4
Autophagy and Prion Disease

Zongbing Hao and Guanghui Wang

Abstract Prion disease, also known as transmissible spongiform encephalopathy (TES), is a fatal neurodegenerative disease caused by prion protein. The most important pathogenesis is related to changes in the conformation of cellular prion proteins (PrP^C). The histopathological features of prion disease are spongiform degeneration, neuronal deficiency, glial activation and the deposition of amyloid-like PrP^{Sc}. Cellular prion protein, ubiquitously expressed in the brain and other tissues, is transformed into the PrP (PrP^{Sc}) isoform in the prion disease. In this chapter, we summarize the research progresses of prion disease, the structural organization and normal function of PrP^C in the central nervous system. Moreover, the formation and transmissibility of prion aggregations (PrP^{Sc}) were also included. But we mainly focused on the function of PrP^{Sc} in autophagy. Several autophagic-related markers, such as p62 and LC3, are significantly upregulated in models of prion disease. Recent advances in the autophagic invention in prion disease and several pharmaceutical targets of autophagy were reviewed in this chapter. It is necessary to understand how the prion protein spread, transport and recycle, and what is the relationship between the clearance and autophagy.

Keywords Prion disease · Autophagy · Cellular prion protein · PrP^{Sc}

4.1 Introduction

Prion diseases include Creutzfeldt-Jakob disease (CJD), fatal familial insomnia (FFI), Gerstmann–Sträussler–Scheinker disease (GSS) and recently discovered variably protease-sensitive prionopathy (VPSPr) in humans, while in animals, prion diseases mainly include scrapie in sheep and goats, chronic wasting disease (CWD) in deer and elk, and bovine spongiform encephalopathy (BSE, mad cow disease). Patients

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with prion diseases have clinical manifestations of progressive dementia, ataxia, muscle spasm, accompanied by symptoms of the pyramidal system and extrapyramidal system. Prion diseases in humans can be divided into three types: autosomal dominant inheritance, spontaneous occurrence and acquisition through environmental exposure to prions.

4.2 Prion Diseases

4.2.1 The History of Prion Diseases

In April 1985, a new disease usually occurring in adult British cattle around 4 years old, namely "mad cow" disease, was discovered by scientists. The symptoms of this disease mainly include abnormal behavior, irritability, sensitivity to sound and touch, especially head touch, late-onset convulsions, increased respiratory rate, weight loss and extreme marasmus. Anatomically it was found that the gray matter of the bovine central nervous system formed a cavernous vacuole, bilateral symmetry lesions on the brain gray matter; the nerve fiber network has a moderate number of discrete ovate and spherical hollow, nerve cell swelling, degeneration and necrosis. In November 1986, the disease was named bovine spongiform encephalopathy. Since then, the disease has spread rapidly and thousands of British cattle suffered from mad cow disease and died.

In fact, the disease was not first discovered. As early as 1730, there was a record of scrapie. In 1958, a study reported that glial cells in the brain of the sheep showed abnormal proliferation, which was considered to be a neurodegenerative disease. In 1913, a maid in a German monastery suffered from mental illness. The patient was cheerful and lively before the onset of the disease, but she became sluggish and faltering after the onset. Since then, the doctor Jacobs of the University of Hamburg published a similar paper in 1921 and named the disease "Creutzfeldt-Jakob" syndrome. In the 1950s, the American pediatrician Gajdusek found that the patients mostly appeared in the corpse tribe, so it was assumed that this was an infectious disease. Moreover, Gajdusek performed an experiment using orangutan and found that the pathological changes of the brain tissue of the orangutan were similar to those of humans. Since Kuru disease can be transmitted, there must be some kind of "pathogen", but the pathogen cannot be sterilized by ultraviolet radiation, indicating that the pathogen is not a nucleic acid, so the pathogen was named "lentivirus" at that time.

As the research progressed, the scientists found that the pathogen is actually a kind of factor that does not contain DNA or RNA, which is composed of protein that can be self-propagating and infectious. It is called prion protein; prion is the meaning of protein virus, and it is the only virus found so far that does not use nucleic acid as a template, but uses protein as a template for infection. The 1997 Nobel Prize in Physiology or Medicine was awarded to the American biochemist

Stanley B. Prusiner for his outstanding contribution to the pathogenic mechanism of prion protein.

4.2.2 Prion and Related Disease

Prion is derived from the abbreviation of proteinaceous infectious particles, which is the meaning of infectious protein particles. During the process of regular protein conversion to prion protein, there is an increase in β-sheet structure and tend to form oligomers, thereby causing neurodegeneration. In mammals, more than a dozen proteins have been found to form prion-like proteins, which cause neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and so on. Because these proteins and prions have similar conformational changes, and the mechanisms causing the disease are very similar, some researchers believe that these proteins should be called prion-like proteins. Most neurodegenerative diseases are age-dependent, and incidence is positively correlated with age, but there is increasing evidence that prion protein may be one of the important factors causing neurodegenerative diseases.

There is also a class of prion proteins in mammals that play a vital role in the body without causing disease. These non-pathological prions include: cytoplasmic polyadenylation element binding (CPEB) protein, mitochondrial antiviral-signaling (MAVS) protein and T-cell-restricted intracellular antigen 1 (TIA1). Pathological prions include prion protein (PrP) and prion-like protein, including amyloid protein (Aβ), tau protein and α-synuclein.

4.3 The Mechanism of Prion Disease

4.3.1 The Function of PrP

4.3.1.1 The Structural Organization of PrP^C

The human PrP^C protein is synthesized from 253 amino acids and is cleaved to 209 amino acids after removal of the N-terminal and C-terminal signal peptides. The N-terminal signal peptide mainly anchors the PrP protein into the lumen of the endoplasmic reticulum for folding. The PrP^C protein is further post-translationally modified in the endoplasmic reticulum and Golgi cavity, including removal of the signal peptide at the C terminus and addition of a GPI anchor (glycosylated phosphatidyl alcohol, GPI), and the PrP protein is further anchored to cell membrane surface by GPI anchor (Stahl et al. 1987). The membrane-localized PrP^C can be further cleaved into two forms, one is to cleave at the N-terminal 110 site, forming a soluble N-terminal fragment, and a C-terminal anchored to the cell membrane. One

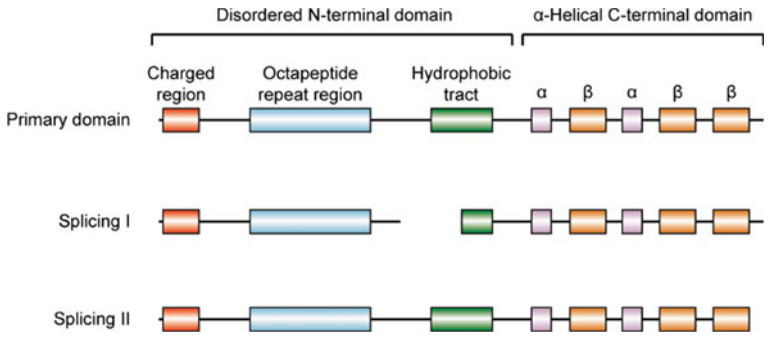

Fig. 4.1 The domain of human PrP^C

near the C-terminal cleavage of the GPI anchor causes PrP^C to detach from the cell membrane, whereas the function of the shed PrP^C remained unclear. PrP^C mainly functions on the cellular membrane in the monomer form. Recent studies found that PrP^C can also be expressed in the mitochondria and nucleus (Bravard et al. 2015; Faris et al. 2017). The structure of PrP^C is mainly divided into a disordered N-terminal domain and a structured C-terminal domain. The N-terminal domain mainly comprises a positively charged motif, a series of four octapeptide repeat sequence and a hydrophobic domain. The C terminus mainly comprises three α-helices and two β-sheets, as well as a C-terminal GPI anchor (Fig. 4.1).

4.3.1.2 The Function of PrP^C

The function of PrP^C protein is mainly studied by various PrP knockout mice. Early studies of a large number of knockout mice found that the function of PrP is related to the formation of myelination of the peripheral nerve (Bremer et al. 2010), synaptic function (Collinge et al. 1994; Lledo et al. 1996), maintenance of hematopoietic stem cells (Steele et al. 2006), immune response after ischemic injury (McLennan et al. 2004) and the function of olfactory bulb (Le Pichon et al. 2009). With the advance in technology of transgenic mice construction, recent research found that some of the early functions may be related to the off-target effect (the expression of the SIRPα protein, which is similar in position to the *Prnp* gene, is knocked down in PrP knockout mice). The latest *(ZH3) PrP−/−* mice model ruled out the possibility of off-targeting and the model found that PrP mainly affects the myelination of peripheral nerves without affecting previously reported synaptic functions (Nuvolone et al. 2016).

4.3.1.3 The Formation of PrP^{Sc}

PrP mainly exists in two forms, one is PrP^{C}, an isoform in normal cells; the other is PrP^{Sc}, which is transformed from PrP^{C} and can be easily aggregated in vivo. Moreover, it is infectious and is the only component of prion protein. In prion diseases, PrP^{Sc} self-propagation with PrP as a template leads to further misfolding and aggregation of the prion proteins.

4.3.2 Prion-Like Protein

4.3.2.1 Aβ, Tau and AD

Since the discovery of Kuru-like syndrome that can be transmitted from humans to chimpanzees, scientists are thinking about whether AD and PD can also be transmitted. In 1993, a study reported that Aβ depositions were found in the brain of marmoset monkeys that were injected with brain homogenates of AD patients after 6–7 years (Baker et al. 1993). Intracerebral and intraperitoneal inoculations with brain homogenate of AD patients were shown to cause Aβ deposition in mice. In addition, Aβ deposition was initially found only in the basal temporal and orbitofrontal neocortex of AD patients. Aβ depositions were later found in brain regions such as neocortex, hippocampus, diencephalon and basal ganglia (Braak and Del Trecidi 2015; Thal et al. 2002). In severe AD patients, Aβ depositions can also be detected in the brainstem and cerebellum. These results indicate that Aβ was transmissible in vivo. Tau, another important AD-causing protein, also has prion-like properties. In the patient's brain, tau nerve fiber aggregation was first discovered in the locus nucleus and the entorhinal cortex, and then in some parts of the neocortex, followed by presence of tau aggregations in large parts of the neocortex (McKee et al. 2013).

4.3.2.2 α-Synuclein and PD

The α-synuclein mutation is an important cause of PD, and α-synuclein aggregation (Lewy body inclusions) was detected in 95% of PD patients. Like Aβ and tau, α-synuclein is also transmissible. At the beginning of the symptoms, α-synuclein-positive Lewy pathological aggregates are mainly in the olfactory bulb, the dorsal motor nucleus of the vagal and glossopharyngeal nerves of the medulla oblongata. Starting from the brainstem, Lewy body spread from the pons to the midbrain and basal forebrain, followed by the neocortex (Braak and Del Tredici 2009). Recent studies found that Lewy body can also be transmitted through the intestinal-brain and brain-intestinal axis systems. And this is very similar to the peripheral-neural spread of prion protein (Goedert et al. 2013).

4.3.3 The Mechanism of Prion Protein Toxicity

4.3.3.1 The Formation of Prion Aggregations

PrP^{Sc} mainly comprises a β-sheet domain, while PrP^{C} mainly comprises an α-helical domain. The PrP^{Sc} structure is resistant to the cleavage of proteolytic enzymes and is thus distinguishable from the PrP^{C} form. In addition, recent studies have found that aggregates in the form of PrP^{Sen} are also transformed from PrP^{C}, but are more sensitive to proteolytic enzymes, mainly in the form of oligomers, and are highly toxic (Sandberg et al. 2014). This is the currently accepted "protein-only" hypothesis that proteins can spread in vivo without the involvement of nucleic acids. Virus-like particles were found in CJD patients and scrapie sheep, and the aggregates can be cell toxicity in vitro and in vivo.

4.3.3.2 The Transmissibility of Prion Protein

An important feature of PrP^{Sc} aggregates is that they are transmissible and infectious. A study found that mice inoculated with prion protein derived from patients showed symptoms associated with prion disease. The transmission of prion protein in a species is much less efficient than transmission between two different species, and this is called transmission barrier (Collinge et al. 1996).

However, the transmission of prion protein has the characteristics of conformational transformation. In fact, this process depends on the structure and sequence of PrP^{C} proteins of different species. If two species have similar PrP^{C} protein structure, the prion protein may keep the original conformation, and if the structure of the PrP^{C} protein is different, the prion protein may shift from one conformation with a large proportion to a type with a small proportion in the process of transmission. And this is important for the sustained transmission of prion protein in vivo.

4.4 The Function of Autophagy in Prion Diseases

4.4.1 The Discovery of Autophagy in Prion Diseases

Autophagic vesicles have been described earlier in brain tissue from CJD patients. In addition, a variety of vesicular structures and autophagosomes appeared in cultured prion protein infected neurons. It has been recently discovered that autophagic vesicles are distributed in the pericytes, axons and synapses of neurons of human prion diseases. Consistent with this result, an obvious upregulation of p62 protein levels was detected in brain of prion-inoculated mouse and infected cultured cells (p62 is an important adaptor protein in autophagy). In addition, aggregated p62 co-localizes with PrP^{Sc} in perinuclear when proteasome was inhibited (Lopez-Perez

et al. 2019). And overexpression of p62 in cells can accelerate the clearance of PrP^{Sc}. The study found that several autophagic proteins significantly changed in the scrapie model. Immunohistochemical staining showed significant p62 positive staining in each brain region of the scrapie model but not in control groups. In addition, upregulated LC3-B was detected in the cerebellar Purkinje cells and basal ganglia neurons of the scrapie model, while the mRNA levels of *Atg9* and *Atg5* in the cerebellum were significantly downregulated, indicating dysregulated autophagy in prion disease (Lopez-Perez et al. 2019).

4.4.2 Mechanisms of Autophagic Activation in Prion Diseases

We already know that autophagy plays a role in prion disease, but how did autophagy is activated? AMPK acts as a serine, threonine protein kinase that regulates the initiation of autophagy by phosphorylating ULK1 at specific sites. The study found that in the disease model of prion protein infected hamsters, two key proteins AMPK and ULK1, which is related to the initiation of autophagy, were significantly upregulated, and AMPK-Thr172 and ULK1-Ser555 were also significantly upregulated. It should be emphasized that AMPK and ULK1 are upregulated in the early stages of prion infection, indicating the importance of autophagy in the pathogenesis of prion diseases. In addition, knockdown of ULK1 in cells can effectively inhibit the lipidation of LC3 (Fan et al. 2015).

4.4.3 The Intervention of Autophagy in Prion Diseases

Histone deacetylase 6 (HDAC6) regulates acetylation of important stress processes-related protein in neurodegenerative diseases, including aggregation, autophagy and apoptosis. The protein levels of HDAC6 were significantly upregulated in primary cortical neuron 3 h after prion protein treatment, followed by decreasing after 24 h, and the localization of HDAC6 in the cells also changed. Inhibition of HDAC6 expression aggravated prion-treated neuronal apoptosis, while overexpression of HDAC6 attenuated neuronal damage. Further studies found that HDAC6 increases intracellular autophagy through the PI3K-Akt-mTOR signaling pathway, promoting the degradation of prion protein in cells, and thus alleviates neuronal apoptosis (Zhu et al. 2016). Caffeine is a psychoactive drug that has been used to increase alertness and energy. It also has a protective effect on Parkinson's disease. Recent studies showed that caffeine also increased intracellular autophagy through Akt signaling pathway and inhibited the neurotoxicity of prion protein (Moon et al. 2014) (Fig. 4.2).

In addition to the regulation of the PI3K-Akt-mTOR autophagy pathway, which can alleviate prion diseases, other signaling pathway-mediated activation of

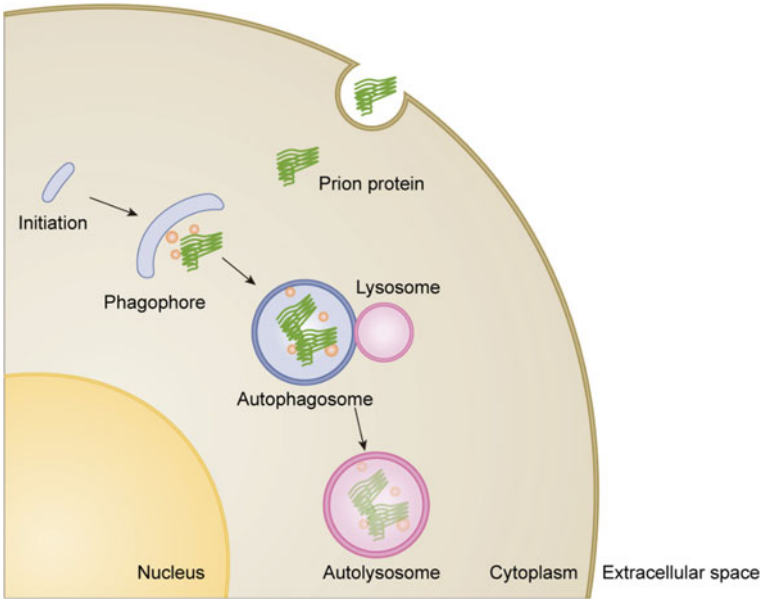

Fig. 4.2 The role of autophagy in the pathogenesis of prion disease

autophagy can also alleviate disease. Sulforaphane (SFN) is an important component of cruciferous vegetables and can significantly reduce the cytotoxicity of PrP (106–126) prion protein, and this effect depends on the autophagy function mediated by AMPK signaling pathway. Drugs that inhibited autophagy or activation of the AMPK signaling pathway counteract the protective effects of sulforaphane (Lee et al. 2014).

In addition to prion diseases, the pathogenesis of neurodegenerative diseases is also associated with mitochondrial dysfunction, and aggregated misfolded proteins can cause mitochondrial dysfunction. It was reported that activated Sirt1 can induce autophagy, thereby protecting neurons by regulating mitochondrial homeostasis and resisting neurotoxicity caused by prion protein. The mechanism is associated with a decrease in membrane potential of mitochondrial and a decrease in fragments of PrP (106–126). Further studies showed that resveratrol, the agonist of Sirt1, can prevent cell damage and oxidative stress from PrP fragment (106–126) treatment, through activating the autophagic pathway (Jeong et al. 2013). E3 ubiquitin ligase Parkin is involved in the degradation of many abnormally folded proteins and is an important intermediate protein in the process of mitochondrial autophagy. The expression of Parkin was significantly downregulated in Neuro-2a cells treated with PrP fragment (106–126) in a time-dependent manner. The Parkin and PrP fragments (106–126) were co-localized in cells. Overexpression of exogenous Parkin attenuated the apoptosis induced by PrP fragment (106–126) and promoted the level of autophagy in cells, with decreased expression of Bax and mitochondrial cytochrome c release

(Khan et al. 2017). These findings suggest that mitophagy also plays an important role in the degradation of prion protein.

4.5 The Complexity of Autophagy in Prion Disease

The function of autophagy in alleviating protein aggregation has reinvigorated the research in neurodegenerative diseases, such as Alzheimer's disease, Huntington's disease and Parkinson's disease. The drugs, which are used to activate autophagy, can reduce the aggregations of PrP in infected neurons and prolong the survival of model mice. The role of autophagy in prion disease is complex, and the activation of autophagy does not always present a protective effect. Previous study reported that pharmacological induction of autophagy by rapamycin inhibited release of exosomes in vitro, however, inhibition of autophagy by wortmannin or using *atg5* knockout cells promotes the release of exosomes. Therefore, although enhanced autophagy can reduce the release of prion protein, autophagy may also promote the release of prion protein at a specific stage. This phenomenon is similar to autophagy in the relationship between cell death and tumors. Autophagy can promote or inhibit cell death or tumor production under different conditions. Therefore, the function of autophagy in prion disease highly depends on the state of the cell, and the way to effectively regulate the autophagic pathway is a key issue in the future.

4.6 Conclusions

Due to the complexity of disease mechanism and autophagy, the relationship between autophagy and prion disease is still not fully understood. For a long time, the drugs for activation of autophagy are effective in inhibiting prion disease. And the neurotoxicity and infectivity of PrP^{Sc} were significantly reduced, which may be caused by increased lysosomal degradation of prion protein. However, its exact molecular mechanism has not been fully elucidated, especially why most of the PrP^{Sc} prion protein is in the endosome and lysosome. So, in future study, it is necessary to understand how the prion protein spread, transport and recycle, and what is the relationship between the clearance and autophagy. In addition, it is more necessary to explain whether the induction of autophagy is mTOR-dependent or independent, or both. Finally, theoretical transformation and its application to anti-prion therapy will be extremely difficult because the link between autophagy and other anti-prion methods is too far. In addition to not being the optimal pharmacokinetic mechanism and possible side effects, blood–brain barrier is one of the biggest obstacles of autophagy treatment.

Further research is needed for the basic biological functions of autophagy in prion diseases. Preliminary data indicates that autophagy plays a key role in regulation of the susceptibility to prion protein, although current studies have difficulty in determining whether autophagy changes are prerequisite for prion protein infectious or

a biological phenomenon post-infection. Similarly, Janus-faced autophagy is essential to the spread of prion protein. The model of yeast prion protein infection and mammalian cell culture indicates that aggregation formation requires the formation of aggregates and fiber ruptures, and whether autophagy is involved in this process would be an interesting direction. Another challenge is to establish a sophisticated mice model that can be used to study the relationship between prion infection and autophagy.

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Chapter 5
Autophagy and Lysosome Storage Disorders

Haigang Ren and Guanghui Wang

Abstract Lysosomal storage disorders (LSDs) are one of the most common human genetic metabolic diseases caused by gene mutations. Up to now, more than 70 LSDs have been identified and mainly divided into five categories. LSDs are mainly caused by defects in the function of enzymes or lysosomal-related proteins in lysosomes, which causes progressive accumulation of undigested macromolecules within the cell and results in stress and dysfunction in cells, tissues and organs. LSDs can result in multiple systemic damages, including the nervous system, skeletal system and reticuloendothelial system, especially in the early stages of the disease. The central nervous system is severely affected. Lysosome is the final degradative organelles for autophagy by which macromolecules and damaged cellular components and organelles are degraded. Impairment in autophagy is a central and common mechanism underlying many LSDs. The modulation of autophagy has been considered as novel therapeutic approach for LSDs.

Keywords Lysosomal storage disorders · Lysosome · Autophagy · Autophagic flux · Combination therapy

5.1 Categories of Lysosomal Storage Disorders

Lysosomal storage disorder (LSD) is a group of hereditary metabolic diseases caused by genetic mutations, of which autosomal recessive inheritance accounts for the majority. Up to now, more than 70 LSDs have been identified. Although the incidence of each type of LSDs is rare, as a group of diseases, LSDs are one of the most common human genetic diseases. The incidence of LSDs in newborns and infants is about 1 in 5000, but the actual incidence may be higher due to the presence of undiagnosed patients or misdiagnosed patients. LSDs are mainly caused by defects in the function of enzymes or lysosomal-related proteins in lysosomes, resulting in the

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substrates not being degraded and accumulating in lysosomes and causing stress and dysfunction of cells, tissues and organs. Although most LSDs are caused by the loss of acidic hydrolase, many patients still suffer from diseases due to the loss of lysosomal membrane proteins or loss of activity of soluble lysosomal proteases. The clinical manifestations of LSDs are diverse and progressive. The common initial pathological manifestation of LSDs is the accumulation of some specific macromolecules or monomeric compounds in the endoplasmic reticulum–autophagy vesicle–lysosomal system. Defects in lysosomal enzyme function are responsible for the aggregation of these molecules and the morbidity in patients (Platt et al. 2018). LSDs can induce multiple systemic diseases, such as the nervous system, skeletal system and reticuloendothelial system. Especially in the early stages of disease, they mainly affect the central nervous system.

The age and symptoms of clinical morbidity in LSD patients vary, depending on the effects of protein function caused by specific gene mutations, the biochemical characteristics of the accumulation and the type of cells in which the accumulation occurs. LSDs are mainly divided into the following categories (Table 5.1): (1) lysosomal enzyme defects: such as aspartylglucosaminuria, mucopolysaccharidoses (MPS), Fabry disease, Gaucher type I, III, III, GM1 gangliosidosis, galactosylceramide lipid storage disease, glycogen storage disease type II and Sandhoff disease; (2) lysosomal enzyme transport defects: mucolipidosis type II and mucolipidosis type IIIA; (3) soluble non-enzymatic lysosomal protein deficiency: Niemann–Pick disease type C2; (4) lysosomal membrane protein deficiency: cystinosis, Danon disease, free sialic acid storage disorder, mucolipidosis IV, Niemann–Pick disease type C1; (5) other lysosomes storage disease: such as neuronal ceroid lipofuscinosis (Platt et al. 2018; Ward et al. 2016).

5.2 LSDs and Autophagy Lysosome System

The occurrence of many LSDs is closely related to lysosomal autonomic dysfunction, a very important acidic organelle in cells, which is a cystic structure surrounded by a single-layer membrane. It contains a variety of acidic hydrolases that can degrade macromolecular substances such as nucleic acids, proteins, lipids, mucopolysaccharides and glycogen to generate small molecules which are transported to out of the cells bypermeases and subsequently reused by cells. The enzymes in the lysosome are all acidic hydrolases, which are synthesized in the Golgi and have the best activity under weakly acidic conditions (pH 5.0). The maintenance of this acidic environment in lysosomes is based on a special transporter-proton pump in the lysosomal membrane that pumps hydrogen ions from the cytosol into the lysosome. Although lysosomes are smaller than endosomes and autophagic vesicles, they are rich in specific transmembrane proteins (mostly glycoproteins) and hydrolases (including proteases, glycosidases, nucleases, phosphatases and lipases). Moreover,with a high buoyant density, the appearance of high electron density can be seen by a projection

Table 5.1 Classification of LSDs

Mechanism of LSD	Disease	Mainly infected organs of the peripheral system	Major defect lysosomal protein
Lysosomal enzyme deficiency	Mucopolysaccharidoses	Cartilage, bone, heart, lung	Mucopolysaccharide
	Aspartylglucosaminuria	Skeleton, connective tissue	Aspartylglucosaminidase
	Gaucher disease type I–III	Spleen, liver, bone marrow	Glucocerebrosidase
	Fabry disease	Kidney, heart	α-galactose
	GM1 gangliosidoses	Kidney, heart	β-galactose
	Galactosylceramide lipid storage disease	Heart	Galactocerebrosidase
	Multiple sulfatase deficiency	Spleen, liver, bone, skin	SUMF1
	Glycogen storage disease type II	Skeletal muscle	α-glucosidase
Lysosomal enzyme transport deficiency	Mucolipidosis type II	Skeleton, heart	*N*-acetylglucosamine phosphoryl transferase α/β
	Mucolipidosis type IIIA	Skeleton, heart	*N*-acetylglucosamine phosphoryl transferase α/β
Defects of soluble non-enzymatic lysosomal proteins	Niemann–Pick disease type C2	Liver	Soluble cholesterol binding protein
Defects of lysosomal membrane proteins	Cystinosis	Kidney, eyes	Cystine
	Danon disease	Heart and skeletal muscle	LAMP-2
	Free sialic acid storage disorder	Liver, spleen, skeleton	Sialyl transporter
	Mucolipidosis IV	Eye	Mucin
	Niemann–Pick disease type C1	Liver	Membrane protein involved in lipid transport
Other LSDs	Neuronal ceroid lipofuscinosis		Genetic defects of other genes

electron microscope, and the degree of acidity and membrane proteins (LMPs) such as LAMP1 and LAMP2 are different from those of the inner body.

When the endosomes and autophagic vesicles are in transient contact with the lysosome, or the contents of the two are exchanged, or directly fused with the lysosome, degradation occurs when the endolysosomal or autophagic lysosome is formed, respectively. Lysosomes are compartments that store acidic hydrolases and enter the cycle of fusion and division with secondary endosomes and autophagic vesicles, while endocytic and autophagic substrate digestion occurs initially in lysosomes. Under physiological conditions, endosomes and autophagosomes are transient organelles. When lysosomal hydrolase, membrane protein or non-enzymatic soluble lysosomal protein is defective, a large number of macromolecules or monomers accumulate in the endolysosomal/autolysosome lysosome, inhibiting those genetically not defective metabolic enzymes and permeases lead to accumulation of secondary substrates, while other LSDs (such as mucopolysaccharidoses I and VI, GM1 gangliosidosis storage disease, etc.) are not due to protease defects but because of the reduced lysosomal protease hydrolysis ability. Accumulation of primary and secondary substrates not only affects the endosome–autophagy vesicle–lysosomal system but also affects other cellular functions, including mitochondria, endoplasmic reticulum, Golgi, peroxisomes and even all cellular functions.

Both endolysosomal and autophagic lysosomes can extend the tubular structure of lysosomal hydrolase and LMPs. At the end of these tubular structures, LC3-negative and LAMP1-positive vesicles bud and acidify and mature into primary lysosomes. A cleavage process can be performed as a lysosomal remodeling in each cycle of endocytic and autophagic substrate degradation, resulting in primary lysosomes that are fused with newly formed endosomes and autophagy vesicles (Xu and Ren 2015).

The detection of exogenous sucrose metabolism in rat kidney fibroblasts fully demonstrates that efficient clearance of endolysosomal/autophagic lysosomal substrates is essential for lysosomal remodeling. Sucrose is a disaccharide composed of monosaccharide glucose and fructose which is difficult to be digested by cells. The sucrose-filled endosomes fuse with lysosomes to form large endosomes, which accumulate in the cytoplasm and appear dense core lysosomes. The absence of, however, the accumulation of sucrose by the ingestion of exogenous invertase promotes the reproduction of dense core lysosomes. Chinese scientists Yu Li et al. found that lysosomes are not regenerated, but are formed by reconstitution or budding of endolysosomal bodies. In patients with sialic acid storage, when lysosomal enzymes are kept in intermediates or common organelles, lysosomal remodeling is deficient, and the patient's fibroblasts lack primary lysosomes (Yu et al. 2010). In addition, studies have shown that damaged lysosomal remodeling can be found in cells which Niemann–Pick disease type C2 (NPC2) is deficient, suggesting that NPC2 protein plays an important role in lysosomal remodeling, but the endosome/autophagy vesicle/lysosomal fusion in the C1 type of Niemann–Pick disease is impaired. In view of the same pathology deficiency of Niemann–Pick disease type C1 and Niemann–Pick disease type C2, it is shown that lysosomal remodeling is as crucial as endosomal/autophagic vesicle/lysosomal fusion.

The mechanisms involved in endolysosomal/autophagy lysosomal clearance dysfunction and lysosomal remodeling defects have demonstrated that the core of this pathway is the mammalian target of rapamycin (mTOR), which is a serine/threonine kinase and governs the synergy of intracellular metabolism and nutritional status. During autophagy, mTOR undergoes a phosphorylation-dependent cycle of inactivation and reactivation, which is required for subsequent formation from lysosomes, which in turn depends on autophagolysosomal substrate degradation and sufficient amino acid levels in the lysosomal cavity. Although there is currently limited information available on lysosomal remodeling and activation of mTOR in LSDs, defects in autophagic lysosomal degradation may impede reactivation of mTOR and thus prevent lysosomal remodeling, resulting in the affected cells being depleted of primary lysosomes. Thus, in addition to the retained autophagolysosome, autophagic vesicles may be retained by defective primary lysosomes, which may also explain the reduction in the level of colocalization of autophagic vesicles and lysosomal markers. Brain model in adolescents with neuronal ceroid lipofuscinosis, fibroblasts of mucopolysaccharidosis type IS, Fabry disease and aspartylglucosaminuria, in patients with NPC1 and NPC2 knockdown human umbilical vein endothelial cells and MCOLN1-deficient Drosophila, the mTOR activity is reduced, while in Sandhoff disease, GM1 gangliosidosis storage disease and in the brain of the C1 type of Mump disease, the activity of mTOR was not reduced. Although the cell biology changes of different LSDs are different, the pathogenesis of LSD is closely related to the autophagy–lysosomal pathway (Settembre et al. 2008).

Autophagic vesicles cause an obstacle to autophagic flux due to a decrease in lysosomal activity in most LSDs. LSD is characterized by the accumulation of substrates to be degraded due to lysosomal defects. Some LSDs are defects in sulfatase activity, and the sulfatase modification factor responsible for post-translational modification (SUMF1) can activate sulfatase, and SUMF1 exhibits functional defects in multiple autosomal recessive diseases and multiple sulfatase deficiency. Ballabio found that sulfatase knockout mice have a similar phenotype to patients with multiple sulfatase deficiency, significant metabolite accumulation in lysosomes, and increased expression of both inflammatory markers and apoptotic markers. On comparison of the pathology of two lysosomal storage model mice (multiple sulfatase deficiency and mucopolysaccharidosis type IIIA), the number of autophagosomes in these mice compared with wild-type mice increased, due to the autophagic vesicle/lysosomal fusion blocked, resulting in autophagic dysfunction, that is a large number of metabolic accumulations in lysosomes. Therefore, LSD can be considered as an autophagic dysfunction disease, which is similar to the pathological mechanism of many neurodegenerative diseases. The cells of LSDs showed a significant increase in the number of LC3- and lysosomal-positive organelles, indicating that the production of autophagosomes remained to a certain extent normal. However, from the large accumulation of LC3-II related to autophagic substrates and autophagosome, the autophagic flux in LSD has obvious obstacles, and the autophagy function is abnormal. In model rats with cathepsin D knockout ($CD^{-/-}$) and cathepsin B and L double knockout ($CB^{-/-}/CL^{-/-}$), abnormal vesicle structures were found to accumulate in brain neurons. It is an autophagosome and contains some

cytoplasmic components. Autophagy vesicle immunofluorescence microscopy and cryo-immunoelectron microscopy showed the membrane-bound LC3 in the stress granules of $CB^{-/-}/CL^{-/-}$ rat brain neurons and axons. There was a large accumulation of LC3-II, and it was localized on the autophagosome membrane, and most of the LC3-positive markers were not LAMP1-positive, indicating that lysosomal dysfunction caused the formation of autophagosomes. Furthermore, as with $CD^{-/-}$ neurons, autofluorescence and subunit c of mitochondrial ATP synthase also accumulate in neurons of $CB^{-/-}/CL^{-/-}$. Therefore, $CD^{-/-}$ mice and $CB^{-/-}/CL^{-/-}$ mice mimicked neuronal ceroid lipofuscinosis/Batten disease. Juvenile neuronal ceroid lipofuscinosis is caused by a mutation in the lysosomal protein CLCN3, which accumulates the mitochondrial ATP synthase subunit c, suggesting an autophagic pathway that regulates damaged mitochondrial degradation is impaired. In an animal model of adolescent neuronal ceroid lipofuscinosis in which *Cln3* and *CbCln3* mutant genes were knocked, cerebellar autophagy markers LC3-II were significantly increased, while mTOR was down-regulated. The isolated autophagic vesicles and lysosomes did not mature in wild-type mice, and the mitochondrial ATP synthase subunit c accumulated in autophagic vesicles, and both LC3 and LAMP1 were mainly localized in their respective organelles. Therefore, cathepsin and mitochondrial ATP synthase subunit c have similar phenotypes to some extent, suggesting that they play similar roles in the autophagy pathway, and both have autophagic vesicles and lysosome fusion disorders, affecting autophagic flux. In a variety of sulfatase deficiency and mucopolysaccharidosis type IIIA lysosomal storage model mice with severe neurodegenerative diseases, autophagic vesicles and lysosomal fusion also have significant obstacles, resulting in the accumulation of phagocytic vesicles, and the damage of the autophagy pathway cannot effectively degrade the exogenous aggregation-prone protein (such as the huntingtin protein with polyglutamine repeat and the mutant α-synuclein), resulting in a large number of ubiquitinated proteins and dysfunctional mitochondria accumulate, causing cell death (Lieberman et al. 2012; Moors et al. 2016; Ward et al. 2016). In addition, the function of lysosomes is dependent on the ability of lysosomal membranes to fuse with other membranes in cells. In lysosomal storage, accumulation of lysosomal substrates and lysosomal dysfunction and endocytic transport obstacles are also relevant. In LSD, cholesterol accumulates on the lysosomal membrane in cells, reducing the ability of lysosomes to fuse with endocytic or autophagic vesicles, and the key component of cell membrane fusion is *N*-ethylmaleimide-sensitive factor attachment protein (SNAP) receptor (SNAREs), which accumulates abnormally in the cholesterol-rich region of the lysosomal membrane of LSD, is locked in the complex, destroying it sorting and looping (Fraldi et al. 2010). Reducing the level of cell membrane cholesterol in LSD can restore normal SNARE function and effective lysosomal fusion function. Therefore, it can be said that LSD is an "autophagic disease" and has a common molecular mechanism with other neurodegenerative diseases (Ward et al. 2016).

5.3 Autophagy and Glycogen Storage Disease Type II and Danon Disease

5.3.1 Glycogen Storage Disease Type II

The glycogen storage disease type II (Pompe disease) is the first identified LSD. This disease is caused by the complete loss of enzyme activity caused by a mutation in the acid maltase, also known as acid α-glucosidase (GAA). Acidic α-glucosidase is a lysosomal glycogen hydrolase whose loss of enzyme activity leads to accumulation of glycogen and autophagosomes in lysosomes, which is also a pathological feature of this type of LSD. In the most severely ill infants and young children, severe weakness and heart failure can occur, and if not treated in time, they will die within one year. Even in progressive late-onset patients, many patients eventually die from respiratory failure.

Enzyme replacement therapy (ERT) with a normal functional human recombinant acid α-glucosidase, which replaces the functional deficient enzyme in patients, is approved for the treatment of Pompe disease. Since the pathogenesis of the disease is thought to be a progressive enlargement of glycogen-filled lysosome that causes it to rupture and release glycogen and toxic substances into the cytosol, early treatment before lysosomes destroyed can reverse the cascade reaction. However, the therapy has a good effect on the myocardium but is ineffective on skeletal muscle. RET therapy is ineffective against skeletal muscle and was first seen in the Pompe disease mouse model of GAA knockout. In the non-effect fast muscle cells, a large amount of autophagosome accumulation was found by electron microscopy, and a large number of clustered lysosomal proteins LAMP1 and LC3 positive vesicles were observed in almost all muscle fibers. As the knockout mice grow older, autophagic vesicles increase. From the morphological point of view, the ultimate destruction of muscle fibers and muscle damage is abnormal autophagy rather than lysosome swelling, and both the production of the autophagy vesicles and autophagy vesicle lysosome fusion were affected, and BECLIN1, GABARAP, ATG7 and LC3 were over-regulated. Thus, the symptomatic phenotype that occurs in the fast muscles of GAA knockout mice is not due to autophagy activation but the autophagic flux inhibition caused by autophagic vesicles and lysosomal fusion. In Pompe muscle fibers, autophagic substrates such as p62/SQSTM1 and polyubiquitinated proteins accumulate, and ubiquitin-positive proteins aggregate earlier than the symptomatic phenotype and parallel with the progression of the disease. In Pompe mice with muscle Atg5 or Atg7-specific defect, glycogen was almost completely cleared after ERT therapy, but this was not seen in control young and old Pompe mice. It is worth noting that the use of ERT therapy and inhibition of autophagy can improve Pompe mice to wild-type mice with muscle-specific autophagy deficiency, although in wild-type mice with muscle-specific autophagy deficiency, the mitochondrial aggregation, mild atrophy and age-dependent decrease in dysfunction were reported, but the health index (life, exercise and monofilament contraction) of these mice was much better than that of Pompe mice. Many muscle cells in human delayed patients (including adolescents

and adults) also exhibit autophagic vesicle accumulation, while lysosomes outside the autophagic vesicle accumulation area are substantially normal. It is further shown that in wild-type animals, autophagy defects cause muscle cell damage, but in Pompe mice, reducing autophagy levels reduces the formation of autophagic vesicles, while slows down the autophagic flux blockade-induced cell damage (Lim et al. 2018).

5.3.2 LAMP2-Deficient Glycogen Storage Disease

Danon disease, also known as lysosomal-associated membrane protein 2 (LAMP2)-deficient glycogen LSD or normal acid maltase LSD, is defective in function of LAMP2. This disease is inherited by the X chromosome and is extremely rare. Its main phenotype is severe cardiomyopathy and skeletal muscle weakness accompanied by mental retardation. Danon's disease is the first reported LSD involving autophagy dysfunction. The accumulation of autophagic vesicles was detected in various tissues of the *Lamp2* knockout mouse model including liver, muscle and myocardium, and the lysosomal degradation of long-time proteins in hepatocytes was decreased. LC3-II positive autophagic vesicle accumulation and large p62 aggregates were also detected in the living tissue of the patient. Mitochondrial clearance mechanism (mitophagy) associated with impaired autophagy function was found to be defective in cardiomyocytes differentiated from iPSC cells from LAMP2 mutant patients and Lamp2-deficient mice (Nascimbeni et al. 2017).

5.4 Autophagy and Mucopolysaccharide Storage

Mucopolysaccharidoses (MPS) are a class of LSDs caused by defects in enzyme function that catalyze the degradation of mucopolysaccharides. Mucopolysaccharides are a long, repetitive complex of sugar chain molecules. When enzymes that catalyze the degradation of mucopolysaccharides are defective, the degradation of the mucopolysaccharides including dermatan sulfate, heparin sulfate, keratan sulfate, chondroitin sulfate or hyaluronan may be inhibited, resulting in their progressive accumulation. The accumulation of mucopolysaccharides in lysosomes can cause dysfunction of cells, tissues and organs. It is known that 11 kinds of functionally deficient enzymes lead to seven different mucopolysaccharidosis diseases, of which type III MPS is also called Sanfilippo syndrome, which is caused by the enzyme deficiency of degradation of heparan sulfate, which can be divided into four different types (A, B, C and D) that are caused by different enzyme functional defects. Among them, MPS type IIIA is caused by defects in the function of heparan N-sulfatase enzyme, which is characterized by severe mental symptoms, hyperactivity and relatively mild physical symptoms (Boudewyn and Walkley 2019; Settembre et al. 2008).

Polysulfatase deficiency (MSD) is a rare but more damaging disease in which patients develop complex multisystem phenotypes due to impaired sulfatase activity. This disease is caused by mutations in the coding gene of sulfatase modifying factor 1 (SUMF1) leading to the necessary post-translational modification of all sulfatase enzymes. The lack of normal activity of sulfatase activity in MSD patients causes accumulation of sulfate and mucopolysaccharide, resulting in the simultaneous clinical symptoms of at least seven diseases, five of which are mucopolysaccharidosis. Most of the phenotypes of these diseases can be mimicked in the Sumf1 knockout MSD mouse model.

Impaired autophagy may play a major role in the pathogenesis of MSD and MPS disease. Compared with wild-type mice, autophagy vesicles increased significantly in multiple brain regions of MSD and MPS IIIA mouse models, and colocalization of LAMP1 and LC3 was decreased in MSD and MPS IIIA model cells, suggesting that autophagy was decreased. There is a barrier to the fusion of vesicles and lysosomes. A large accumulation of autophagic substrates such as polyubiquitinated proteins and dysfunctional mitochondria also indicate autophagic dysfunction in MSD and MPS IIIA mice. In addition, MSD cells have a markedly reduced ability to degrade exogenous proteins such as mutant huntingtin and mutant α-synuclein, which can cause Huntington's disease and Parkinson's disease, respectively. During skeletal development, chondrocytes in MSD mice have lysosomal storage and clearance of autophagic vesicles leads to changes in energy metabolism and cell death (Fecarotta et al. 2018).

In MSD and MPS IIIA, the distribution of membrane lipids and SNARE protein was abnormal, and significant cholesterol accumulation appeared on the lysosomal membrane. In vitro, wild-type cells loaded with cholesterol showed a similar phenotype to MSD cells: autophagy vesicles and lysosome fusion were blocked, while methyl β-cyclodextrin reduced cholesterol and restored lysosomal normal function. SNARE protein is an important component in the cell membrane fusion mechanism. There is a large amount of abnormally chelated SNARE protein in the cholesterol-rich region on the lysosomal membrane of LSD. This abnormal distribution affects the normal function and recycling of SNARE protein, which directly affects the fusion ability of lysosomal membrane.

MPS type VI mucopolysaccharidosis, also known as Maroteaux-Lamy syndrome, is caused by a deficiency in the lysosomal enzyme *N*-acetyl-4-sulfatase (arylsulfatase B, ARSB). ARSB catalyzes the hydrolysis of sulfates in mucopolysaccharides, mainly dermatan sulfate. When the ARSB enzyme activity is absent, dermatan sulfate cannot degrade and accumulates in different cells and tissues. The clinical features of MPS VI are rough face, short stature, multiple osteogenesis abnormalities, impaired joint function and strength, hepatosplenomegaly, abnormal heart valves and corneal opacity. Although there are scattered damaged nerve cells in the animal model of ARSB activity deficiency, there is no clinical evidence that the central nervous system of patients with MPS VI is affected. Using fibroblasts from patients with MPS VI, it was found that lysosomal storage leads to impaired autophagy and accumulation of polyubiquitinated proteins and dysfunctional mitochondria, but LC3 has no effect on colocalization with LAMP2, indicating that the fusion of autophagic vesicles

with lysosomes was not completely blocked, probably because mucopolysaccharide inhibited lysosomal cathepsin activity and caused autophagy damage (Fecarotta et al. 2018).

5.5 Autophagy and Sphingolipidosis

Sphingolipidosis is a type of hereditary disease that often affects the nervous system due to disorders of sphingolipid metabolism. These diseases occur mainly in children and result in neurodegenerative diseases producing bradykinesia and myoclonus, and affecting white matter leads to weakness and paralysis. Sphingolipids are a class of lipids enriched by the nervous system and are critical for the development and function of the nervous system. Degradation of sphingolipids requires multiple steps and relies on multiple lysosomal hydrolases. Sphingolipidosis is the accumulation of fully or partially undegraded sphingolipids due to functional hydrolytic enzyme deficiencies. Patients present with sphingomyelin, glycolipid, glucocerebroside, ganglioside, unesterified cholesterol and sulfur compound accumulation. Recent studies have found that the addition of glycosphingolipids to the cell's culture medium induces autophagy, but autophagic vesicle clearance is reduced and rapidly causes accumulation of autophagic vesicles. Sphingolipid storage diseases include Niemann–Pick disease, Gaucher disease, Fabry disease and GM1/2 gangliosidosis. Autophagy changes were detected in some sphingolipid storage diseases, the most widely studied of which was Niemann–Pick type C1 disease (NPC1) (Platt 2014).

5.5.1 Niemann–Pick Disease Type C

Niemann–Pick disease is divided into A, B, C1 and C2 types, in which type A and type B are caused by loss of acid sphingomyelinase activity due to mutation of *SMPD1*; and type C1 and type C2 are caused by mutation of NPC1 or NPC2 gene, which encoding the two proteins are thought to act synergistically to activate cholesterol efflux. In Niemann–Pick type C disease (NPC), 95% of patients are of NPC1 mutations and 5% of NPC2. NPC1 is a multiple transmembrane protein that contains a sterol-sensitive domain similar to the domains of cholesterol synthesis regulators such as HMG-CoA reductase, SCAP and 7DHCR. NPC2 is a soluble protein localized in the late endosomal/lysosomal cavity that binds cholesterol and extracts cholesterol from the lipid bilayer and transports it to another bilayer or NPC1 amino-terminal domain. Because of the defective function of NPC1 or NPC2, low-density lipoprotein (LDL)-cholesterol receptor-mediated endocytosis of unesterified cholesterol is widely accumulated in cells.

In the brains of *Npc1* mutant mice and in neurons differentiated by patient-derived iPSC, significant accumulation of autophagic vesicles and blockade of autophagic flux were observed, and this autophagy disorder resulted in significant

cell viability decline. The mutated NPC1 prevents cholesterol from escaping from the compartment of the endolysosomal, deactivating SNARE function and inhibiting autophagosome maturation. Similar to the NPC1, accumulation of autophagosomes and autophagy substrates and decreased lysosomal activity were also found in NPC2-knockdown adipocytes. Treatment of wild-type fibroblasts with a small molecule, U18666A, which induces NPC-like lipid transport defects, also resulted in up-regulation of BECLIN1 and LC3-II, increased p62 and polyubiquitinated proteins, suggesting that lipid transport can be blocked. These studies have shown that autophagy regulation disorders are an important mechanism of NPC, and autophagy is a potentially valuable therapeutic target for NPC.

5.5.2 Gaucher Disease

Gaucher disease is the most common type of sphingolipidosis caused by mutations in the last step of glucosamine glucosidase (GCase) or its activator saposin C. Autophagosome maturation defects, TFEB down-regulation, and decreased lysosomal gene expression were detected in neurons differentiated from patient-specific iPSCs. A similar phenomenon of impaired autophagocytic degradation occurs in fibroblasts of patients with saposin C mutations, which is associated with decreased cathepsin B/D activity. Glucocerebrosidase V394L mutant homozygous mice and saposin C mutant mice are often used as animal models of Gaucher disease, which show punctate p62 aggregates in their neurons and astrocytes, while undigested materials are found in the axonal vesicles with accumulation of autophagic substrates. Recently, in the Drosophila Gaucher disease model, which has knocked out glucocerebrosidase, severe lysosomal defects and autophagic flux arrest have also been observed, resulting in shortened lifespan, neurodegeneration, which are associated with disorders of the mTOR signaling pathway, which can be reversed by rapamycin. These findings indicate a defect in the intracellular autophagy degradation function of patients with Gaucher disease or model animals (Aflaki et al. 2017).

5.5.3 Fabry Disease and GM1 Gangliosidosis

GM1 gangliosidosis is a common autosomal-negative genetic lipid storage disease caused by lysosomal β-galactosidase mutation leading to GM1-ganglioside deposition. The main phenotype is dysfunction of the central nervous system, visceral hypertrophy and skeletal dysplasia. Fabry disease is caused by the accumulation of lysosomal α-galactosidase A, which causes its substrate acyl sheath satriletrihexose to accumulate in the body. The basal level of autophagosome marker LC3-II was significantly increased in the GM1 gangliosidosis disease model, and autophagy vesicles, BECLIN1 expression and the abnormal mitochondria and so on were found in GM1-gangliosidosis model mice. The basal level of LC3 and the LC3 level after

starvation treatment in the cells of Fabry patients were significantly higher than those of normal cells. Treatment of starved fibroblasts and lymphocytes from Fabry patients with lysosomal protease inhibitors showed impaired autophagic flux, and the autophagy degradation pathway was more severely damaged than several other sphingolipidosis. In addition, the increase in p62 and ubiquitinated substrate proteins in kidney tissue and fibroblasts in Fabry patients further suggests impairment of autophagy degradation (Patterson 2013).

5.6 Autophagy and Mucolipidosis Type IV

Mucolipidosis type IV (MLIV) is an autosomal recessive disorder characterized by acute psychiatric symptoms and visual abnormalities, including retinal degeneration, corneal opacity, optic atrophy and strabismus. Heterogeneous inclusion bodies are found in many tissues of MLIV patients, including multi-layer wafer complexes formed from lipids and mucopolysaccharides, as well as soluble granular proteins. MLIV is caused by a mutation in MCOLN1 (mucolipin1, also known as TRPML1), a cation channel protein of the lysosomal inner membrane, an inwardly corrected ion channel (from the lysosomal cavity to the cytosol) that contributes to Ca^{2+}, Na^{+}, K^{+} and Fe^{2+}/Mn^{2+} permeation membranes.

MCOLN1 mediates the efflux of Ca^{2+} from endosomes and lysosomes, maintaining organelle homeostasis and fusion of endosomal vesicles. MCOLN1-mediated Ca^{2+} release activates the Ca^{2+}-dependent phosphatase calcineurin, which promotes dephosphorylation-mediated nuclear translocation of TFEB and activates expression of some lysosomal proteins and autophagy-related proteins. Degradation of autophagic vesicles in fibroblasts from patients with MLIV and the fusion of autophagic vesicles with endosomes/lysosomes were blocked, accumulation of autophagic vesicles occurred, and p62 aggregation and abnormal mitochondria appeared. Activation of MCONL1 by overexpression or pharmacological methods can promote autophagic flux. In addition, MCONL1 interacts with the chaperones HSPA80 and DNAJB1 and is involved in the regulation of molecular chaperone-mediated autophagy (CMA). CMA deficiency and decreased levels of LAMP2A are found in MLIV fibroblasts leading to an increase in oxidized protein. MCOLN1 forms a heterodimer with its family proteins MCOLN2 and MCONL3. Both MCOLN3 knockdown and MCONL3 mutants overexpressing the death channel domain defect can inhibit starvation-induced autophagy, suggesting that MCOLN dimer plays an important role in autophagy regulation. *Mcoln1*$^{-/-}$ mice showed no significant behavioral and morphological changes compared with the littermate wild-type mice born, but with age, *Mcoln1*$^{-/-}$ mice showed progressive limb weakness, eventually quadriplegia and death in the eighth month. In the brain of 8-month-old *Mcoln1*$^{-/-}$ mice, lysosomal-labeled inclusion bodies were found in neurons, astrocytes, oligodendrocytes, microglia and endothelial cells, indicating that MCOLN1 deficiency inducing autophagy disorders play an important role in neurodegeneration (Boudewyn and Walkley 2019).

5.7 Potential Treatments for LSDs

In the development of different LSDs, different symptom severity, different tissues and different stacked molecular types involve autophagy. Although there are differences between some diseases and the actual sample of patients tested, it is often seen that the autophagic flux is blocked (Table 5.2). In one aspect, secondary accumulation of autophagic substrates such as polyubiquitin, p62 and abnormal mitochondria is increased, and on the other hand, an increase in autophagic vesicle-forming factors such as BECN1 attempt to compensate for impaired autophagic flux. In some LSDs, abnormal activation of mTOR can also cause obstruction of autophagosome lysosome formation. Therefore, LSD can be called autophagy disease, and there are indications that changing autophagy function or enhancing lysosomal function can be used as a potential treatment for LSD. Impaired autophagy and accumulation of autophagic substrates in lysosomal storage indicate that some of the pathogenic mechanisms of lysosomal storage are similar to those caused by other autophagy defects. Especially in some neurodegenerative diseases affecting the elderly, such as Parkinson's disease, Alzheimer's disease and Huntington's disease, there are many aggregate proteins in

Table 5.2 Summary of research on autophagy in LSD

Type of disease		Autophagic vacuole accumulation	Defective autophagic vacuole degradation	Increased autophagic vacuole formation	Increased poly-ub proteins	Increased p62
Glycogen metabolic disease	Pompe disease	Y	Y	Y	Y	Y
	Danon disease	Y	Y	N	N	N
Mucopolysaccharidosis	Multiple sulfatase deficiency	Y	Y	N	Y	Y
	Mucolipidosis type IIIA	Y	Y	N	Y	Y
	Mucolipidosis type VIA	Y	Y	N	Y	Y
Sphingolipidoses	Niemann–Pick disease type C	Y	Y	Y	Y	Y
	Gaucher disease	Y	N	N	Y	N
	Fabry disease	Y	Y	N	Y	Y
	GM1 gangliosidoses	Y	N	Y	N	N
Mucolipid storage disease	Mucolipidosis type II	Y	N	N	Y	Y
	Mucolipidosis type III	Y	N	N	Y	Y
	Mucolipidosis type IV	Y	Y	Y	Y	Y
Ceroid lipofuscinosis	CLN10	Y	N	N	N	N
	CLN3	Y	N	Y	N	N

their nerve cells, and these aggregate proteins are the key elements of the cause cascade. Further links between LSDs and neurodegenerative diseases are found in LSDs such as NPC, mucopolysaccharidosis and sphingolipidosis caused by defects in amyloid degradation. Phosphorylated tau protein aggregation and neurofibrillary tangles, both are commonly detected in Alzheimer's disease. Neuropathology of NPC disease and some protein aggregations, for example, including phosphorylated tau protein aggregation and α-synuclein aggregation, are strikingly similar between neurodegenerative diseases in which amyloid β aggregates disease. Recent studies have found that lysosomal acidification is impaired in a mouse model of Alzheimer's disease, and lysosomal loss in a mouse model of Parkinson's disease further indicates a link between lysosomal dysfunction and neurodegenerative diseases. Since a large number of studies have attempted to use autophagy as a target for the treatment of neurodegenerative diseases, these studies have considerable reference for the treatment of LSDs (Marques and Saftig 2019; Moors et al. 2016).

Many researchers are aware of the important role of autophagy pathways that maintain normal protein recycling in these diseases. For example, drug stimulation to activate autophagy can prevent protein aggregation in neurological diseases such as Huntington's disease. In the Huntington's drosophila model, rapamycin was shown to prevent neurodegeneration, while in the Huntington's mouse model, neuropathological status was improved by enhancing autophagy to clear the accumulation of pathological huntingtin. Rapamycin also removes aggregated proteins containing aggregating proteins such as polyglutamine or polyalanine, while at the same time improves the animal's phenotype. Trehalose is a disaccharide present in many non-mammals, which is an mTOR-independent autophagy activator that exhibits anti-apoptotic effects in cultured neurons and can also accelerate mutant huntingtin and clearance of α-synuclein. However, in most LSDs, autophagic vesicle formation is not defective, but the autophagic flux is blocked. Therefore, activating the autophagy scheme has some adverse effects. The effective efficacy of inhibiting autophagy after ERT therapy in Pompe disease indicates that inhibition of autophagic vesicle formation is a possible therapeutic strategy in the case of autophagic flux block.

The important transcription factor TFEB that targets lysosomal activation and autophagy pathway activation is considered to be a very promising therapeutic strategy. TFEB is a very important nuclear transcription factor that regulates the formation and function of endosomes, autophagic vesicles and lysosomes. It regulates the expression of hundreds of lysosomal genes and regulates the expression of CLEAR genes. Subsequent studies have found that TFEB can not only regulate the production of lysosomes but also play a very important role in the regulation of autophagy. At present, the methods that may be of potential concern to diseases in the academic community are: by activating the TFEB gene, enhancing the entire lysosomal and autophagy pathways, and promoting autophagic flux. Since LSD is essentially caused by lysosomal and autophagy dysfunction, rational regulation and improvement of lysosomal and autophagy functions may be beneficial for the treatment of LSDs. Since autophagy plays an important role in controlling protein quality and maintaining cell homeostasis, autophagy disorders can lead to a variety of diseases including lysosomal storage, so autophagy can be enhanced by rational regulation

of TFEB expression. In particular, reducing the blockage of autophagic flux may be beneficial to alleviate LSDs caused by autophagy dysfunction (Sardiello 2016).

Recent studies have shown that the accumulation of cholesterol in the lysosomal inner membrane can alter the organization and composition of lysosomes and reduce their ability to fuse. Some drugs, such as methyl-β-cyclodextrin, restore lysosomal fusion by reducing cholesterol levels on the membrane, and can also restore LSDs such as blocked autophagic flux in MSD and MPS IIIA. Although such compounds are quite toxic in vivo, the FDA has approved the clinical use of a number of cyclodextrins such as Kleptose, Trappsol and CAPTISOL. Experiments have shown that NPC mice can reduce cholesterol and ganglioside deposition after long-term treatment with hydroxypropyl-β-cyclodextrin, and the level of autophagy is normal and significantly improves the survival rate of mice. But no similar effects were observed in MPS IIIA and GM1-mucopolysaccharidosis mice. The therapeutic effectiveness of autophagy-regulating drugs at different stages of the autophagy pathway, and to observe the phenotypic improvement of cells and animal models of LSDs need further exploration, which will help to understand LSDs and develop effective treatment.

5.8 Conclusions

Lysosomal storage disorders are characterized by accumulation of macromolecules that are not degradable, including functional defects in lysosomal hydrolases, or by vesicle trafficking, autophagosome maturation, lysosomal acidification or lysosomal membrane transport molecules. The abnormality of the endosomal lysosome formation process is abnormal. Each lysosomal storage subtype exhibits several different lysosomal biological functional defects, and to some extent requires different therapeutic strategies to cope. Autophagic flux block is a distinctive feature of many LSDs, suggesting that we stimulate autophagy and reduce autophagic flux block which is a clinically viable option that can be used as a common LSDs therapeutic targets. However, since the current study only found that autophagy inducers are only effective for very few LSDs, the autophagy regulation mechanism in LSDs needs more in-depth research and evaluation. Combination therapy is a very promising treatment strategy for LSD. The autophagy induction combined with ERT therapy has shown very good efficacy, but this therapy is only suitable for individual subtypes of LSDs and is expensive, need to develop new therapeutic strategies that are cheaper and suitable for more subtypes of LSDs, such as TFEB and other therapeutic targets. Different combination therapies may be required for different lysosomal storage subtypes, but the primary role of autophagy regulators is not negligible.

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Chapter 6
Autophagy and Mitochondrial Encephalomyopathies

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Abstract Mitochondrial encephalomyopathies are a group of disorders affecting skeletal muscles and brain. Although the symptoms vary among these disorders, mitochondrial DNA mutation or loss is the common characteristic. The abnormality of mitochondrial genome usually causes the dysfunction of mitochondrial respiratory and even mitochondrial damage. As a critical way of degradation, attention has been paid to the involvement of autophagy in encephalomyopathies. Autophagy is found activated in these encephalomyopathies-relevant cells as a compensatory manner to eliminate these damaged and dysfunctional mitochondria. However, accumulating evidences indicate that autophagy is incompetent to clear them. The insufficient mitophagy may ultimately accelerate cell death. Here we discuss the involvement of autophagy in encephalomyopathies based on the current evidence. We further look into the future to rescue encephalomyopathies by regulating autophagy. Only five encephalomyopathies are included in this chapter due to the availability of evidence. Nevertheless, these encephalomyopathies share a variety of common features and autophagy may also be regulated in the other encephalomyopathies.

Keywords Encephalomyopathy · Autophagy · Mitochondria · Mitophagy

6.1 Introduction

Mitochondrial diseases refer to a variety of disorders caused by mitochondrial dysfunctions. Most of the mitochondrial diseases can be attributed to mutations of genomic DNA, while a small portion (approximately 15%) is due to the mutations of mitochondrial DNA (mtDNA) (Dimauro and Davidzon 2005). Given the abundance of mitochondria in muscles and nervous system, mitochondrial dysfunctions are prone to cause disorders in skeletal muscle and brain, which is termed mitochondrial encephalomyopathy (ME). The prominent symptoms of ME include growth

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and mental retardation, dystonia, myasthenia, visual impairment, hearing loss, dysfunction of autonomic nervous system and cognition, and so on. The ME can be divided into several specific disorders or symptoms:

(1) Mitochondrial myopathy, encephalomyopathy, lactic acidosis, stroke-like symptoms (MELAS);
(2) Myoclonic epilepsy with ragged red fibers (MERRF);
(3) Kearns–Sayre syndrome (KSS);
(4) Chronic progressive external ophthalmoplegia (CPEO);
(5) Leigh syndrome;
(6) Alpers' disease;
(7) Leber's hereditary optic neuropathy;
(8) Neuropathy, ataxia, retinitis pigmentosa and ptosis (NARP);
(9) Myoneurogenic gastrointestinal encephalopathy (MNGIE).

The pathologies of ME remain not fully elucidated. Nevertheless, it has been widely accepted that mitochondrial dysfunction plays a central role. Advances in this field revealed the involvement of autophagy in ME. Autophagy-related elimination of dysfunctional mitochondrial can be a novel strategy for ME therapy.

6.2 MELAS Syndrome

Mitochondrial myopathy, encephalomyopathy, lactic acidosis, stroke-like symptom (MELAS) is an encephalomyopathy characterized by myasthenia, pain and seizure. In most cases, the patients suffered from lactic acidosis, fatigue, vomiting, somatic pain and stroke-like episodes. It is postulated that the stroke-like episode could be associated with the cerebrovascular disorders caused by mitochondrial dysfunction. The individuals affected showed temporary hemiparesis and blurred vision. Repeated stroke-like episodes can progressively lead to vision loss, dyskinesia and dementia. MELAS is a maternal inherited disease caused by mutant mitochondria-encoded genes (Zeviani et al. 1993). There are at least 39 mutations related with MELAS, although there are also some non-inherited cases. The well-studied mutant genes include NADH dehydrogenase and several genes encode tRNAs. More than 80% of MELAS individuals carry the mutation of mitochondrial genome A3243G which encodes $tRNA^{Leu(UUR)}$ (Goto et al. 1990; Ikeda et al. 2018). This mutation causes the abnormality of protein translation and synthesis. A3243G mutation led to mitochondrial membrane potential loss and respiratory inhibition in fibroblast cells (Cotan et al. 2011; Ogle et al. 1997). In neurons, this mutation impaired the function of mitochondrial Complex I and subsequently caused oxidative stress and reduced ATP level (Hamalainen et al. 2013). Emerging study indicated that in the fibroblast cells derived from MELAS patient carrying A3243G mutation, fatty acids could not be utilized as energy sources for mitochondria which might aggravate the intracellular bioenergy crisis (Lin et al. 2017).

It has been reported that autophagy-related genes, including *ATG5*, *ATG12, beclin1* and *LC3*, were upregulated in the fibroblasts obtained from MELAS patients. Imaging analysis characterized mitochondria engulfed by autophagosomes in these cells, which accumulated with lysosomes (Cotan et al. 2011). The abnormality of mitochondrial elimination could be attributed to the insufficient of ATP and subsequent disability of fusing between autophagosomes and lysosomes. Collectively, autophagic flux could be blocked in these cells. Autophagy blockage by pharmacological approaches and silencing ATG5 led to apoptosis of MELAS fibroblasts, suggesting the benefits of autophagy (Cotan et al. 2011). Mitophagy deficit can be critical for the pathology of MELAS. Compared with the normal control, the MELAS fibroblasts showed equivalent mitochondrial content and remarkable 3–4-fold increase of mutant mitochondrial tRNA (James et al. 1996). Similar alternations were also found in neurons. In the iPS-derived neurons with MELAS mutation, the functions of mitochondrial Complex I was significantly lost. Moreover, PINK1 and Parkin, the mitophagy-related proteins, showed mitochondrial recruitment, indicating that MELAS neurons attempted to eliminate the impaired mitochondria via autophagy machinery (Hamalainen et al. 2013). These data further highlighted the important contributions of mitophagy in the process of MELAS (Table 6.1). The mitochondrial dysfunction in MELAS cells may be related to reduced co-enzyme Q (CoQ) (Lopez et al. 2014). Supplement of CoQ partly reversed the oxidative stress, mitochondrial depolarization and Complex I dysfunction in MELAS cells, suggesting that the CoQ can be a potential therapy (Chen et al. 2011; Garrido-Maraver et al. 2012). Interestingly, CoQ even reversed the dysregulations of autophagy in MELAS cells (Rodriguez-Hernandez et al. 2009). Overall, mitophagy deficit may cause excessive accumulation of impaired mitochondria which in turn lead to further mitophagy deficit. This positive feedback manner could act as a deleterious driving force to exacerbate MELAS syndrome.

6.3 MERRF Syndrome

Myoclonic epilepsy with ragged red fibers (MERRF) is an encephalomyopathy with maternal inherited mutations of mitochondrial DNA. The individuals suffering from MERRF usually showed myoclonic epilepsy, cerebellar ataxia and weakness of proximal limbs. There are four mutations in mitochondrial DNA that have been identified to cause MERRF. The A8344G mutation in mitochondrial genome encoding $tRNA^{Lysine}$ is the most common one. The A8344G mutation reduces the activities of both Complex I and IV in mitochondrial respiratory chain. Paucity of ATP, depolarization and excessive ROS production are documented in the affected mitochondria (De la Mata et al. 2012).

Evidence from immunohistochemistry and electromicroscopy supported increased number of autophagosomes in skeletal myocytes of MERRF patients, suggesting autophagy activation (Yuan et al. 2013). In consistent with this observation, cells carrying A8344G mutation exhibited decreased activities of Complex I, II, III

Table 6.1 Autophagy in mitochondrial encephalomyopathies

Syndrome	Related mutation	Alteration in autophagy	References
MELAS	m.3243A>G	Upregulated autophagy-related genes (*ATG5*, *ATG12*, *beclin1*, *LC3*), accumulated mitochondria engulfed by autophagosomes, mitochondrial recruitment of PINK1 and Parkin	Cotan et al. (2011) and Hamalainen et al. (2013)
MERRF	m.8344A>G	Increased number of autophagosomes, upregulated autophagy-related genes (*ATG12*, *beclin1*, *LC3*), increased conversion from LC3-I to LC3-II, increased formation of ATG12-ATG5 complex, colocalization of LC3 and mitochondria	Yuan et al. (2013), De la Mata et al. (2012) and Wang et al. (2014)
LHON	m.12338T>C	Downregulated level of LC3, accumulated p62	Zhang et al. (2018)
	m.11778G>A, m.3460G>A	Impairment of autophagy activation, mitochondrial accumulation	Sharma et al. (2019)
KSS	mtDNA depletion	Increased expression of ATG12, inhibition of mTOR	Alemi et al. (2007)
CPEO	m.7486G>A	Lower ATP lever, mitochondrial membrane potential loss, protein precipitation	Bacalhau et al. (2018)

and IV, while the expressions of autophagy-related genes, namely *ATG12, beclin1* and *LC3*, were upregulated. Additionally, increased conversion from LC3-I to LC3-II and formation of ATG12–ATG5 complex also implied the activation of mitophagy in cells with A8344G mutation (De la Mata et al. 2012; Wang et al. 2014). Indeed, LC3 is prone to colocalize with fragmented, rather than tubular mitochondria, suggesting the elimination of mitochondria in autophagy-dependent manner (De la Mata et al. 2012) (Table 6.1). Furthermore, in hybrid cells representing MERRF, the mitochondrial outer membrane protein voltage-dependent anion channel (VDAC) showed increased vulnerability to oxidative damage (Wu et al. 2010). Parkin-dependent ubiquitination of VDAC was well documented in mitophagy process. These studies suggested that ROS derived from damaged mitochondria may induce mitophagy with the involvement of Parkin, which may subsequently help to clear impaired mitochondria

and thus attenuate apoptosis. Overall, mitophagy activation may inhibit the apoptotic demise of MERRF cells.

In contrast to the observation aforementioned, the heat shock protein 27 (Hsp27) degraded by autophagy machinery in cells carrying A8344G mutation (Chen et al. 2011). Hsp27 is a critical protective protein which responses to stress. Overexpression of Hsp27 protected hybrid MERRF cells from UV light-induced apoptosis. Likewise, overexpression of carbonic anhydrase-related protein VIII (CA8) reduced cyclosporine-caused death of hybrid MERRF cells. However, like the case of Hsp27, CA8 was also degraded by autophagy in MERRF cells (Wang et al. 2014). Collectively, these studies indicated the autophagic loss of protective proteins in MERRF cells. Autophagy therefore seems to act like a double-edge sword in the pathology of MERRF, and the selectivity of autophagy cargo may decide the contribution of autophagy to MERRF.

6.4 Leber's Hereditary Optic Neuropathy

Leber's hereditary optic neuropathy (LHON) is the first documented human disorder known to be caused by mitochondrial DNA mutation. Clinically, there is an acute or subacute onset of visual loss of both eyes without pain complaint. There are at least 18 mis-sense mutations within nine mitochondrial genes that could lead to the phenotypes of LHON directly or indirectly. The G11778A is the most frequent mutation of LHON (Huoponen et al. 1991), which impaired the mitochondrial Complex I activity and reduced ATP production. Emerging study also revealed that the T12338C mutation causes downregulated level of LC3 and accumulates p62, which suggested that T12338C mutation impaired the mitophagy besides its other deleterious effect on mitochondrial functions (Zhang et al. 2018) (Table 6.1).

Significant autophagy impairment and mitochondrial accumulation have been documented in the hybrid cells carrying G11778A mutation. Administration of rapamycin, an autophagy activator, reversed the low intracellular ATP and promoted cell survival (Sharma et al. 2019; Dai et al. 2014). Rapamycin significantly promoted the elimination of mitochondria and reduced the copy number of mutant but not total mitochondrial DNA (Dai et al. 2014). In addition, imaging study revealed increased colocalization of mitochondria with autophagosomes, indicating that rapamycin might enhance mitophagy besides its activation of autophagic flux (Sharma et al. 2019). These studies indicated that rapamycin could be a potential drug for LHON therapy.

6.5 KSS and CPEO Syndrome

Kearns-Sayre syndrome (KSS) has a typical onset before 20 years of age. The individuals suffering from KSS are characterized by degenerative paralysis of muscles

controlling eyes and eyelids movement, which resulted in ptosis and ophthalmoplegia. Some patients may also have other symptoms, including heart block, ataxia, gravis and deafness. Chronic progressive external ophthalmoplegia (CPEO) showed similar syndromes as KSS, yet in a less extent, which include gravis, loss of muscle endurance and ptosis and so on (Houshmand et al. 2006). These two diseases share similar phenotypes and pathological mechanisms; hence they are included within one section here.

It has been accepted that KSS is caused by a variety of mitochondrial DNA (mtDNA) loss. The most common loss is the missing 4.9 kb base-pair sequence from 8469 to 13147 in mtDNA. Nevertheless, it remains largely unclear how the mtDNA loss relates with the phenotypes of KSS. There are studies indicating that mtDNA loss ultimately leads to the dysfunction of oxidative phosphorylation in mitochondria, inhibition of protein translation and misfolding of mitochondrial proteins (Alemi et al. 2007). The misfolded proteins blocked and impaired the ubiquitin–proteasome system (UPS). The loss of mtDNA triggers the expression of ATG12, which might be activated as a compensation for the impairment of UPS and subsequent low intracellular amino acid. The mTOR-eIF2α signaling pathway was assumed to be involved in this (Alemi et al. 2007) (Table 6.1). At present, there is a paucity of data describing autophagy regulation in KSS, and it remains not elucidated whether autophagy is a mechanism underlying KSS.

There is an emerging report indicating two CPEO families whose members carrying the mutation of OPA1 showing parkinsonism and dementia (Carelli et al. 2015). OPA1 is a protein responsible for mitochondrial fusion. The fused mitochondria are not prone to be eliminated by autophagy. Nevertheless, it is unclear whether the phenotypes of these patients were associated with mitophagy deficit. Additionally, a novel mutation in mtDNA (m.7486G>A) has been identified in CPEO patients. This mutant gene encodes $tRNA^{Ser(UCN)}$, and the fibroblasts derived from patients had a lower ATP level and mitochondrial membrane potential accompanied with protein precipitation (Bacalhau et al. 2018). These alternations suggested the autophagy dysfunction (Table 6.1).

6.6 Conclusions

Although it remains not fully elucidated, current evidence suggested that autophagy can be a common mechanism underlying these mitochondrial encephalomyopathies. The mutations or loss of mtDNA cause mitochondrial dysfunction and trigger autophagy via multiple pathways. Among them, the dysfunction of Complex I could be a critical factor. Lines of evidence indicated that Complex I dysfunction will lead to ROS production and ATP reduction, which are well-documented factors inducing autophagy activation. Noteworthy, the activated autophagy may be incompetent to clear damaged mitochondria due to some possible reasons. (1) mitochondria accumulation exceeds the elimination capacity of autophagy machinery; (2) excessive or misfolded mitochondrial proteins occupy the autophagy machinery; (3) low ATP

level may hamper autophagy because of its energy-consuming nature. Besides these disorders, there are several myopathies not related with mitochondrial DNA mutations, for example, Ullrich congenital muscular dystrophy, Bethlem myopathy and congenital myosclerosis, which also show mitochondrial dysfunctions and involvement of autophagy (Bernardi and Bonaldo 2013). Therefore, the current studies support the hypothesis that inefficient autophagy, in particular mitophagy, could be a common feature underlying these disorders. Nevertheless, it remains challenging to promote the efficiency of autophagy and thus alleviate cellular dysfunctions in these diseases.

To enhance the CoQ10 could be a potential way. Reduced CoQ10 was found in a variety of mitochondrial encephalomyopathies, which directly lead to excessive oxidative stress (Rodriguez et al. 2007; Lopez et al. 2014). Intriguingly, supplement of CoQ10 partly reversed the insufficiency of mitophagy (Rodriguez-Hernandez et al. 2009). These observations suggesting the potential therapeutic values of CoQ10 might raise a novel regulation manner of mitophagy, although its mechanisms need further address. Taken together, enhanced autophagy or mitophagy may provide a novel therapy for these mitochondrial encephalomyopathies in the future.

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Chapter 7
Autophagy and Ischemic Stroke

Yanlin Zhang, Yongjun Cao, and Chunfeng Liu

Abstract Ischemic stroke refers to brain tissue ischemia, hypoxic necrosis, and brain softening caused by the interruption of the blood supply to the brain without adequate collateral circulation, thus resulting in neurological symptoms. Autophagy is activated in various cell types in the brain, such as neurons, glial cells, and microvascular cells, upon ischemic stroke. Autophagy efflux injury plays an important role in this pathologic process. This chapter outlines the induction of basal autophagy, autophagy in neurons, and the crosstalk between autophagy, necrosis, and apoptosis that contributes to ischemic stroke. We will highlight the interactions between autophagy, oxidative stress, endoplasmic reticulum stress, and mitochondrial dysfunction, and the role of autophagy in ischemic stroke. We will also review the recent advances in the understanding of the involvement of autophagy in the pathological process of cerebral ischemic preconditioning, periconditioning, and postconditioning.

Keywords Ischemic stroke · Autophagy · Apoptosis · Necrosis · Self-adaption

7.1 Introduction

Ischemic stroke refers to brain tissue ischemia, hypoxic necrosis, and brain softening caused by the interruption of the blood supply to the brain without adequate collateral circulation, thus resulting in neurological symptoms that, in addition to whole-brain ischemia and hypoxic necrosis, include choking, irregular heartbeat, and global brain lesions caused by apnea. Atherothrombotic cerebral infarction, cerebral embolism, and lacunar infarction are the most common types of ischemic stroke. Among these types, atherothrombotic cerebral infarction accounts for 60–80% of ischemic stroke. The onset is relatively fast, often reaching a peak in minutes, hours, or even 1–2 days. The treatment of acute cerebral infarction is closely related to the time window. Acute cerebral infarction can be divided into three stages: the super early stage (within 1–6 h of onset), the acute stage (1–2 weeks after onset), and recovery period

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(>2 weeks to 6 months after onset). Focusing on treating the ultra-early and acute phases is particularly important for the prognosis of patients. Treatment for these phases mainly includes returning blood supply to the cerebral ischemic area as soon as possible, preventing ischemic brain edema, and neuroprotection.

Despite many attempts, current treatments for ischemic stroke are still limited, and ischemic stroke remains one of the leading causes of death and disability, particularly in adults (Lakhan et al. 2009). Reperfusion and neuroprotection are two strategies for treating ischemic stroke. Autophagy is involved in the protein degradation process in many cells, and there is increasing evidence that the activation of autophagy is a neuroprotective mechanism in patients with ischemic stroke. The signaling pathway associated with autophagy is a promising target for the treatment of ischemic stroke, but the exact role of autophagy activation in ischemic events and its potential value in drug therapy remain to be affirmed.

Cerebral damage in ischemic stroke begins in childbirth and can cause permanent brain damage, leading to cognitive and motor dysfunction. The degree of ischemia and hypoxia depends on the degree of maturity of the brain and the duration of the damage (Lakhan et al. 2009). Based on the pathophysiological knowledge of stroke, two treatment strategies are used for irreversible cerebral infarction caused by the ischemic cascade. The first goal is to restore blood flow to the damaged area with thrombolytic, antithrombotic, and antiplatelet aggregation drugs. To date, the only drug approved for clinical thrombolytic therapy is rt-PA. However, its use is subject to an extremely strict time limit of 3–6 h, and it is associated with a high risk of bleeding complications. The second goal of treatment is neuroprotection, specifically by increasing endogenous cytoprotective responses and reducing the activation of metabolic pathways leading to cell death to prevent the death of nerve cells. The concept of neuroprotection in ischemic stroke is related to the presence of the ischemic penumbra. In neuroimaging, the ischemic penumbra is the region that surrounds the ischemic center and separates it from the healthy tissue. In the ischemic penumbra, blood flow is gradually reduced (but at a slower rate than that at which the blood flow to the ischemic center is reduced), resulting in metabolic disorders and the activation of many molecular pathways. Therefore, the ischemic penumbra is an area considered to be recoverable. However, over time, the cells in the ischemic penumbra also die, and the extent of the ischemic center expands. Novel and effective neuroprotective strategies inhibit the expansion of the ischemic center and extend the potential for reperfusion therapy (Green and Shuaib 2006). A reduction in the survival time of the ischemic penumbra and in inflammation is caused by ischemia and the damage is induced by reperfusion (Fisher 2011).

The entire process of autophagy is highly conserved in evolution, and autophagy is the process by which cells and cytokines that are to be catabolized are self-consumed. In this complex metabolic process, which involves the endosomal–lysosomal system, excessive, old, and unwanted cytoplasmic components (including long-lived proteins) and organelles, mitochondria, peroxisomes, Golgi proteins, and endoplasm are digested by lysosomal enzymes (Lakhan et al. 2009). Autophagy occurs during the normal growth and differentiation of tissue cells and under pathological conditions such as starvation and bacterial infection and due to misfolded proteins and organelle

damage (Kuma et al. 2004). Autophagy is widely believed to be a self-protecting cellular catabolic pathway through which some long-lived or misfolded proteins and damaged organelles are degraded and circulated to maintain cellular homeostasis (Thorburn 2008). Many studies have demonstrated that autophagy protects cells from death by inhibiting apoptosis. In addition to preventing apoptosis to inhibit cell death, autophagy also causes excessive cell death. Therefore, autophagy is called type II programmed cell death to distinguish it from type I programmed death, or apoptosis. Autophagy seems to be a double-edged sword. Whether autophagy is beneficial or deleterious depends on the rate of autophagy induction and the duration of autophagy activation. Recent evidence has shown that autophagy is activated in various cell types in the brain, such as neurons, glial cells, and brain microvascular cells, upon ischemic stroke. Autophagy efflux injury plays an important role in this pathologic process. This chapter outlines the induction of basal autophagy and autophagy in neurons, and the crosstalk between autophagy, necrosis, and apoptosis that contributes to ischemic stroke. We will highlight the interactions between autophagy, oxidative stress, endoplasmic reticulum stress, and mitochondrial dysfunction, and the role of autophagy in ischemic stroke. We will also review the recent advances in the understanding of the involvement of autophagy in the pathological process during cerebral ischemic preconditioning, periconditioning, and postconditioning.

7.2 Basal Autophagy in Neurons

Autophagy can be divided into two categories, namely, basal and induced, according to its effects (Uchiyama et al. 2001). Autophagy is maintained at a low basal level in most cells. From a physiological point of view, autophagy is a fundamental component of the turnover of cytoplasmic components and the selective clearance of damaged organelles such as mitochondria and peroxisomes (Lakhan et al. 2009).

The clearance of unnecessary cells during embryonic development is also an example of the role of basal autophagy, and basal autophagy is also considered a determinant of longevity. The basal autophagy level in neurons determines the maintenance of axonal homeostasis by transporting autophagosomes to neuronal cell bodies through reverse axoplasm and fusion with lysosomes. Conversely, autophagy is also induced under pathological conditions (such as stress and injury), resulting in the synthesis of a large number of autophagosomes and the accumulation of autophagosomes in axons. The degradation of autophagosomes in axons requires entry into lysosomes, which are eventually degraded by cis transport. In addition, impaired autophagy and transport mechanisms in axons can lead to the accumulation of proteins, organelles, and damaged membrane structures in axons, leading to axonal degeneration.

It is generally believed that, under normal conditions, the biosynthesis rate of autophagosomes in neurons is not high, and the low level of autophagy in neuronal cells is related to their ability to utilize glucose and ketone energy materials (Boland and Nixon 2006). When adjacent astrocytes are extremely deficient in energy (such

as during ischemia), sugar is metabolized to lactate and glycogen, which provide a short-term energy for nerve cells. Moreover, nerve cells secrete growth factors and neuropeptides, which have protective effects on nerves. The low levels of autophagy in neurons may be the result of neuronal-specific proteins and protein modifications. One of these proteins is microtubule-associated protein 1B (MAP1B), which binds to light chain (LC3) and is closely related to the formation of autophagosomes. However, there is also a view that the basal autophagy level in neurons is similar to the level of autophagy in other cell types, but newly formed autophagosomes rapidly fuse with lysosomes to eliminate them, thus preventing the formation of autophagy intermediates. It is also almost impossible to detect autophagosomes (Boland et al. 2008).

A lack of basal autophagy is involved in the pathogenesis of a variety of neurodegenerative diseases, as evidenced by recent studies in transgenic mice lacking Atg5 expression in neuronal cells. Rats lacking Atg5 exhibit slow growth and progressive motor and behavioral deficits. Moreover, in the cytoplasm of cells from the thalamus, pons, medulla, and dorsal root ganglion of mice lacking Atg7, large aggregates, ubiquitination (a mark of misfolded proteins), aggregates of inclusion bodies, significant cerebral cortical atrophy, and neurodegenerative changes are observed. These data strongly suggest that the use of basal autophagy to effectively eliminate unwanted proteins in the cytoplasm can prevent the accumulation of abnormal proteins that disrupt the function of the nerves and ultimately lead to neurodegenerative diseases.

7.3 Induction of Autophagy in Neurons

Autophagy in the central nervous system can be induced not only by the ischemic penumbra of the brain but also by nutrient deficiencies, neurotoxins, excitotoxic stimuli, closed head injuries, and neuropathogenic pathways. Accumulating evidence has shown that autophagy is activated in various cell types in the brain, such as neurons, glial cells, and brain microvascular cells, upon ischemic stroke. Autophagy is activated under stress conditions and aids cell survival by controlling the clearance and reuse of intracellular components by activating autophagy–lysosomal degradation mechanisms. In the process of autophagy induction, the regulation of autophagy by neurons is relaxed, and the autophagy transitions from the basal level to the induction level are accompanied by the enhancement of autophagy biosynthesis (Yang and Klionsky 2009).

7.3.1 Neuronal Autophagy Is Controlled by Regulatory Factors in Some Cellular Signaling Pathways

These signaling pathways enable the transition from basal levels of autophagy to high levels, and the induction of these regulators depends on the availability of the nutrient supply.

7.3.1.1 DJ-1/PARK4 and Tp53 Proteins

The DJ-1/PARK4 and Tp53 proteins are considered to be potential regulators of neuronal autophagy. Primary cultures of cerebral cortical neurons have demonstrated that the insulin signaling pathway is involved in autophagy. A study that used an in vitro model of Parkinson's disease showed that E64D mutations in the DJ-1/PARK7 gene reduce basal autophagy, leading to the accumulation of dysfunctional mitochondria (Krebiehl et al. 2010). The Tp53 protein has two functions. Under normal metabolic it is a negative regulator; however, when in response to acute stress, it stimulates Tp53 and causes autophagy in human and murine cell lines, specifically, neuroblastoma cells, and SH-SY5Y cells. The basic role of Tp53 is to inhibit autophagy; however, Tp53 acts as an inducer of autophagy when cells are exposed to nutrient-deficient, carcinogenic, or toxic environments.

7.3.1.2 MTOR Protein

The regulation of autophagy by insulin, amino acids, and AMP kinase (AMPK) is influenced by the serine/threonine protein kinase mTOR. mTOR is generally considered to be a negative regulator of autophagy, and mTOR plays multiple roles in the central nervous system, including regulating cell viability, cell differentiation, transcription, translation, protein degradation, actin cytoskeleton remodeling, and autophagy. In mammalian cells, mTOR assembles into two functionally significant protein complexes: mTORC1 and mTORC2. mTORC1 is a regulator of autophagy and is particularly sensitive to rapamycin (mTOR-specific). mTORC2 is not sensitive to rapamycin, and its main role is to regulate the remodeling of the actin cytoskeleton and the activity of PKCα and AKT kinase. Both of these complexes comprise the mTOR kinase and the MLST8/G[β] protein. mTORC1 contains Raptor, and mTORC2 contains Rictor and mSin1 (Wullschleger et al. 2006).

The mechanism underlying the negative regulating activity of mTOR on autophagy remains unclear. However, after stimulation with insulin, the PI3K pathway plays an important role in the activation of mTOR. PI3K promotes the production of PIP3, and PI3K sequentially recruits PDK1 and AKT kinase to the membrane (Wullschleger et al. 2006). AKT activation is achieved by the phosphorylation of two sites: one is a specific threonine residue that is phosphorylated by PDK1 (Thr308), and the other is a serine residue specific to the mTORC2 complex (S473) (Sarbassov

et al. 2005). The activation of AKT kinase phosphorylation inhibits the activity of the Hamartin (TSC1) and Tuberin (TSC2) protein complex (TSC1/TSC2), a complex that is a negative regulator of mTOR and inhibits the activity of the GTPase activity of the GTPase Rheb (an analog of Ras). The gene product of TSC1, Hamartin, plays a key role in the activation of autophagy in ischemic stroke. Numerous studies have shown that Rheb-GTP can significantly activate mTOR activity both in vivo and in vitro. In addition to being activated by the insulin signaling pathway, mTOR can also be activated by amino acids, which are activated by a reduction in Rheb-GTPase activity (Codogno et al. 2005). Therefore, it is generally believed that the activation of the PI3K pathway through the insulin signaling pathway and of pathways through the direct phosphorylation of amino acids requires the activation of mTOR activity and the concomitant inhibition of autophagy.

In contrast to the insulin and amino acid pathways, activated AMPK kinase activates autophagy by inhibiting the mTOR signaling pathway. AMPK acts as a receptor for cellular energy and regulates metabolic pathways by promoting the production of ATP by catabolic pathways and preventing ATP depletion. This ensures the optimization of the cellular energy process and survival in the face of external disturbances.

In response to an increase in cytoplasmic AMP levels and/or a decrease in ATP production, AMPK is activated by specific upstream kinases that target threonine residues (Thr172), namely LKB1 and CaMKKβ, which activate autophagy by inhibiting the mTORC1 complex. AMPK inhibits mTORC1 by phosphorylating at least two proteins, namely TSC2, which has different serine residues than those targeted by other upstream kinases, and Raptor, which is a partner of mTOR (Li and McCullough 2010). In addition, recent studies have indicated that AMPK-activated autophagy is directly related to the ATG protein. Further studies have found that, in the absence of glucose, AMPK activates autophagy by phosphorylating Ulk1 at the Ser317 and Ser777 sites to activate it. The involvement of the regulation of AMPK in autophagy has also been demonstrated by in vitro experiments, and it has been found that autophagy caused by glucosaccharide deficiency is also inhibited when AMPK is inhibited. Moreover, an inhibitor of AMPK can effectively inhibit autophagy caused by Ca^{2+} (Høyer-Hansen et al. 2007). The phosphorylation of AMPK induces autophagy in the brain and reduces the cerebral infarction volume, neurological deficits, and neuronal apoptosis.

7.3.2 Endoplasmic Reticulum Stress and Mitochondrial Dysfunction Are Important Mechanisms of Neuronal Autophagy Induction

7.3.2.1 Endoplasmic Reticulum Stress

Autophagy caused by cerebral ischemia/hypoxia is associated with the induction of endoplasmic reticulum stress by these conditions (Høyer-Hansen et al. 2007). An increasing number of studies have shown that endoplasmic reticulum stress is an inducer of autophagy. The impairment of endoplasmic reticulum function, including posttranscriptional modifications, a reduction in protein folding control, and the accumulation of misfolded proteins in the ER, leads to ER stress and is considered to induce the unfolded protein response (UPR). ER stress and autophagy are primarily associated with three related signaling pathways, including the PERK pathway, the activation of transcription factor 6 (ATF6), and the inositol-requiring enzyme 1α (IRE1α) pathway. It is now widely accepted that two UPR signaling pathways contribute to autophagy caused by endoplasmic reticulum stress, namely, the PERK/eIF2a pathway and the IRE1/TRAF2/JNK pathway. eIF2a (eukaryotic initiation factor 2α) induces autophagy by inducing the expression of Atg12 to transform LC3-I to LC3-II. Increased Ca^{2+} may be associated with autophagy caused by endoplasmic reticulum stress via the CaMKK/AMPK/mTORC1 pathway (Høyer-Hansen et al. 2007).

Endoplasmic reticulum stress-activated autophagy may be inhibited by the anti-apoptotic protein Bcl-2, which is located on the endoplasmic reticulum membrane. There are at least two hypotheses regarding this mechanism of inhibition. One hypothesis is that Bcl-2 inhibits autophagy by reducing Ca^{2+} levels in the endoplasmic reticulum, thereby reducing the stimulation caused by Ca^{2+} flow, which prevents CaMKK activation and the activation of the CaMKK/AMPK/mTORC1 pathway. The second hypothesis is that Bcl-2 acts directly with beclin1 to reduce the formation of autophagosomes and ultimately leads to the incomplete formation of PI3K complexes (Høyer-Hansen et al. 2007). The ER stress inhibitor salubrinal inhibits autophagy and neuroprotection induced by cerebral ischemic preconditioning. Endoplasmic reticulum stress is strongly associated with various diseases, including inflammatory diseases, autoimmune diseases, and cerebral ischemic injury.

7.3.2.2 Mitochondrial Dysfunction

There is also a close relationship between mitochondrial dysfunction and autophagy. Available evidence suggests that mitochondria are a major target for ischemic injury. Mitochondria play key roles in various cellular processes, including ATP production, Ca^{2+} homeostasis, ROS production and accumulation, and apoptosis. Mitochondrial dysfunction is associated with the excessive production of reactive oxygen species

(ROS), which leads to the activation of autophagy and to stroke, trauma, and neurodegenerative diseases. Excessive ROS can cause DNA damage, lipid peroxidation, and increased rupture/permeability of the lysosomal membrane. Lysosomal membrane rupture/permeability causes the release of cathepsin from the lysosomal lumen and an association with proapoptotic Bcl-2 family members (Bax, Bak, tBid), thereby activating the mitochondrial apoptotic pathway, the release of cytochrome c, and the activation of proteases and DNase II (Tardy et al. 2006). ROS may also affect the molecular mechanisms of autophagy. The production of hydrogen peroxide by oxidative stress causes the inactivation of the cysteine protease Atg4 to induce the formation of autophagosomes, thereby promoting the conversion of LC3, which is a very important step in the process of autophagy.

7.4 Relationship Between Autophagy, Apoptosis, and Necrosis in Ischemic Brain Tissue

Brain damage induced by ischemia results from massive cell death caused by clots or thrombi that destroy the ischemic area. Along with the day-to-day loss of blood flow, the ischemic cascade, which involves a series of biochemical events that lead to the degradation of cell membrane function and structure and ultimately to neuronal death, is initiated (Lakhan et al. 2009).

Patients with acute ischemic stroke lose 120 million neurons per hour (Lakhan et al. 2009). The death of ischemic cells is mainly controlled by apoptotic and necrotic pathways both in vivo and in vitro. These two modes of cell death are significantly different at both the morphological and molecular levels, and intracellular ATP levels are key factors for determining cell fate. Necrosis represents a passive form of cell death that occurs when cells are exposed to acute damage or extreme energy deficiency. Necrosis can cause the swelling of the organelles and the loss of membrane integrity, triggering multiple inflammatory factors and causing damage to adjacent tissues. It has been recently suggested that necrosis is a chaotic and unregulated process that may be regulated by mitochondrial dysfunction, an increased production of ROS, hydrolysis of ATP-depleted calcium-activating enzymes and cathepsins, and by rupture of early plasma membranes (Golstein and Kroemer 2007). Cell necrosis due to stroke mainly occurs in the center of the ischemic area and is mainly due to limited blood flow and harmful substances released by neighboring cells.

The morphological characteristics of apoptosis include nuclear pyknosis, DNA fragmentation, and subsequent cell disintegration into small membrane-wrapped fragments called apoptotic bodies. Apoptosis occurs when ATP levels are sufficient to maintain the function of the appropriate ion channel and ensure cell integrity. Apoptosis does not accompany inflammatory reactions or damage to surrounding tissues. After stroke, apoptosis is mainly present in the ischemic penumbra, and the tissue in this region is much less affected than the ischemic core by the lack of oxygen and nutrients (Broughton et al. 2009).

To understand the pathophysiological processes of cerebral ischemia, Sharp et al. (2000) proposed the differentiation of the ischemic penumbra from several other areas, namely, the selective neuronal death zone, or the area adjacent to the lesion; the protein denaturation zone, or the area in which heat shock proteins appear; the area in which long-term perfusion and reperfusion defects occur, or the area in which hypoxia-inducible factor 1 is expressed and the closest to the outside; and the area of depolarization potential, in which repeated ischemia induces a large number of early genes, specifically c-fos.

The mechanism involved in the death of ischemic cells is as complex as the original kinetics. Necrosis and apoptosis are mechanically and morphologically different, and there is a clear interaction between them in ischemic stroke. In addition, autophagy shares common molecular mediators with necrosis and apoptosis, such as AMPK, Bcl-2, and p62. Cells in the ischemic region express autophagy-related proteins such as beclin1 and LC3. In an experimental model of focal transient cerebral ischemia, the formation of a large number of autophagosomes and the expression of autophagosomes can be observed. The upregulation of autophagy caused by ischemia can be observed in the cerebral cortex, hippocampus, and striatum in animals. Primary cultured cerebral cortical neurons and serum-deprived HT22 cells (a hippocampal cell line) exhibit the same phenomenon (Meloni et al. 2011).

The nuances of the effect of ischemia on nerve cells are due to the simultaneous activation of apoptosis and autophagy pathways by some cells. In a rat model of transient ischemic attack, both the cortical and striatal boundaries exhibit elevated beclin1 levels and the activation of caspase-3. Carloni et al. recently observed beclin-1-positive and TUNEL-positive cells in neonatal rats with hypoxia following the ligation of the right common carotid. Apoptosis and necrosis have been found to occur primarily in the superficial layers of the cerebral cortex. In the deep part of the cortex and in the hippocampal CA1 area, there are fewer cells that express markers of both processes. Importantly, only a few cells positive for beclin1 and propidium iodide (markers of necrosis) are found in the CA1 and CA2 regions of the hippocampus, suggesting that increased protein expression is primarily responsible for the apoptotic pathway (Carloni et al. 2008).

Increased levels of autophagy and apoptosis in neurons are observed in a rat model of severe perinatal ischemia. The autophagy–apoptosis relationship depends on the region of the brain; in the cortex, the relationship between the two is closely linked, and in the hippocampus, the two are relatively independent. Some scholars have suggested that autophagy is a type of apoptosis and is accompanied by an increase in beclin1 and the activation of caspase-3.

There is much evidence that autophagy and apoptosis share molecular inducers and regulatory mechanisms. Moreover, there are many overlaps between autophagic and apoptotic proteins in the autophagy and apoptotic pathways (Maiuri et al. 2007). Atg5 plays an important role in autophagy and increases the sensitivity of apoptosis to stimuli. Moreover, calpain regulates the breakdown of Atg5 to promote the release of cytochrome c and the activation of proteases to cause autophagy and apoptosis. Additionally, beclin1 has a BH3 region and interacts with the antiapoptotic protein Bcl-2. Interestingly, Bcl-2 interacts with beclin1 to inhibit autophagy (Maiuri et al.

2007). Recent studies have shown that apoptotic proteins such as beclin1 not only inhibit autophagy but also promote apoptosis by promoting the release of apoptotic factors from mitochondria. Moreover, recent studies by Grishchuk et al. have shown that beclin1-independent autophagy has an effect on neuronal apoptosis that is dependent on both apoptosis and apoptosis-independent proteases.

The ultrastructural observation of a rat model of stroke induced by middle cerebral artery occlusion revealed that necrotic neuronal death occurs in the early central ischemic region after stroke and that apoptotic and autophagic nerves appear in the peri-ischemic region. Autophagy occurs before apoptosis, and the two do not occur simultaneously in the same dying neuron. Cell necrosis in the early central ischemic region is accompanied by organelle swelling and calpain activation; subsequent apoptosis in the peri-ischemic region is accompanied by early calpain activation-mediated cell division and then by caspase-3- and caspase-9-mediated cell division and the nuclear transcription of apoptosis inducing factor (AIF). The degree of autophagic flow in neurons around the ischemic area after cerebral ischemia begins to increase from 6 h to 24 h after injury. It is worth noting that, after stroke, the inhibitors of apoptosis, namely Z-VAD-fmk and Q-VD-OPH, are not effective in reducing the stroke volume, whereas the intraventricular injection of the autophagy inhibitor 3-MA, even after stroke, significantly reduces (by approximately 46%) the stroke volume. Atg7 knockdown also protects against hippocampal neuronal damage following ischemic stroke. The mechanism by which 3-MA plays a protective role may be related to its inhibition of autophagic and apoptotic cell death, as it has been found that 3-MA can also inhibit caspase activation and AIF nuclear transcription after stroke. In addition, studies that inhibited autophagy by 3-MA or Atg7 silencing demonstrated enhanced cytochrome c release and downstream neuronal apoptosis activation in ischemia/reperfusion-induced OGD and MCAO models. 3-MA and the Akt inhibitor wortmannin significantly inhibit autophagy and decrease apoptosis- and necrosis-mediated cell death by decreasing beclin1 expression, suggesting that autophagy is a part of the signaling pathway that promotes cell survival. Therefore, we speculate that autophagy, apoptosis, and necrosis occur in the ischemic region and that these three processes lead to complex biochemical changes and morphological features of cell death. Autophagy may be a key factor in determining neuronal survival, and modulators of autophagy are expected to be a new option for preventing neuronal death in acute ischemic stroke (Fig. 7.1).

7.5 The Role of Autophagy in Acute Cerebral Ischemia

The current knowledge does not clearly answer whether ischemia-induced autophagy promotes neuronal death, is it an endogenous neuroprotective mechanism, or is merely a collateral phenomenon of apoptosis and necrosis (Rami and Kogel.2008). The exact role and molecular mechanisms of autophagy that are implicated in ischemic stroke have yet to be elucidated. Recent research data show that autophagy plays a major role in acute ischemic stroke in the following seven aspects.

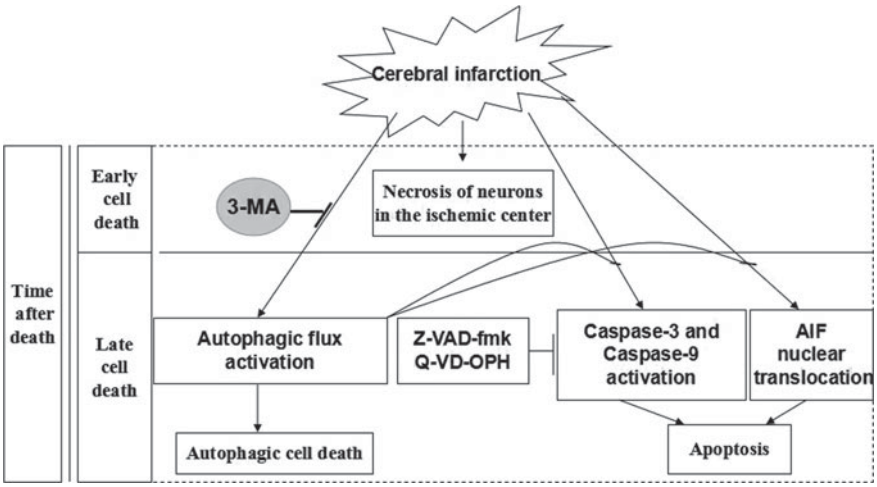

Fig. 7.1 Neuronal death pathway after ischemic stroke. After stroke, necrotic death occurs in neurons in the early ischemic center, followed by apoptotic and autophagic death in neurons in the peripheral ischemic area. The autophagy inhibitor 3-MA can inhibit autophagic and apoptotic neuronal death

7.5.1 Autophagy May Lead to Cerebral Ischemic Axonal Degeneration

A recent study confirmed this view. In rats, a large number of cortical neuron axons degenerate rapidly after 24 h of ischemia/hypoxia. AMPK-mediated autophagy not only enlarges the ischemic area but also worsens the consequences (Du et al. 2009). Neuron-specific Atg7 knockout rats ($Atg7^{flox/flox}$; nestin-Cre) underwent the occlusion of the left common carotid artery for a week and were then exposed to hypoxic treatment, and the hippocampus was observed. The loss of pyramidal neurons was reduced, and the ischemic lesions were significantly reduced. The inhibition of autophagy with drugs can also alleviate focal cerebral ischemia-related nerve damage in rats. Moreover, studies have shown that a single intraventricular injection of 3-MA, an inhibitor of VPS34, significantly reduces the infarct area immediately after the permanent occlusion of the middle cerebral artery in rats (Koike et al. 2008). A similar study that used an in vitro ischemia model found that the autophagy inhibitor 3-MA significantly improves the viability of cortical neurons. Recently, studies have also shown that 3-MA inhibits autophagy in mice with severe cerebral ischemia to prevent the programmed necrosis of hippocampal CA1 neurons, thereby contributing to neuroprotection. Some neuroprotective agents, such as lithium and glial cell line-derived neurotrophic factors, have strong antiautophagic and antiapoptotic effects (Shang et al. 2010). These data suggest that the negative effects of autophagy in cerebral ischemia may be related to the interaction between autophagy and necrosis.

7.5.2 *Autophagy Plays an Important Role in Protecting Neurons from Ischemia-Induced Death*

The inhibition of autophagy with 3-MA or bafilomycin A1 (an inhibitor of autophagosomal and lysosomal fusion) can significantly increase the level of caspase-3 cleavage and the mortality of the hippocampal cell line HT22 under conditions of serum deprivation. In contrast, in a neonatal rat model of H/I-induced brain injury, after treatment with rapamycin, an inducer of autophagy, the expression of beclin1 in the cerebral cortex and hippocampus is increased, and necrotic cell death and brain damage are reduced (Carloni et al. 2008). The beneficial effects of rapamycin have also been observed in a rat model of traumatic brain injury. The neuroprotective effect of rapamycin may be related to the phosphorylation and activation of the PI3K/Akt/mTOR pathway and the transcription factor cAMP response element-binding protein (CREB) (Carloni et al. 2010). Treatment with simvastatin in neonatal mice increases the expression of beclin1 and long-term neuroprotection (Balduini et al. 2009), suggesting that the neuroprotective effect of simvastatin after stroke may be related to the upregulation of autophagy. The induction of autophagy can increase neuronal cell viability in a rat model of poison-induce MCAO by inhibiting apoptosis.

Under abnormal ischemic conditions, β-arrestin-1 is an important scaffold protein involved in membrane receptor desensitization and interacts with it. Beclin1 and VPS34 form preautophagosomal complexes in neurons. The deletion of β-arrestin-1 significantly disrupts the interaction between beclin1 and VPS34 and then blocks autophagosome formation following ischemic stress. At the same time, the absence of β-arrestin-1 enhances neuronal apoptosis, as evidenced by an increase in TUNEL-positive cells and caspase-3.

Autophagy may also be a mechanism of neuroprotection by melatonin. In addition, treatment with SB216763, an inhibitor of the serine/threonine kinase GSK-3β (which plays an important role in neurodegenerative changes), increases autophagic activity in the cortex of mice with permanent middle cerebral artery occlusion, thereby inhibits neuroinflammation (Zhou et al. 2011). At present, the inhibition of the clearance of damaged mitochondria (mitochondrial autophagy) and subsequent apoptosis may be the most important mechanism underlying the role of autophagy in neuroprotection during cerebral ischemia. It has been reported that mitochondria are selectively cleared after the inhibition of proteases, and therefore, the isolation of organelles can explain the mechanism by which apoptosis is inhibited. However, the autophagolysosomal inhibitor bafilomycin A1 can partially inhibit mitochondrial clearance, and there is also strong support for the participation of autophagy in this process. The clearance of damaged mitochondria by autophagolysosomes may be an adaptive metabolic response mediated by hypoxia-inducible factor-1 (HIF-1). Therefore, it is currently believed that mitochondrial autophagy induced by hypoxic treatment is an adaptive mechanism required to maintain redox homeostasis and cell survival (Semenza 2008). However, both mitochondria and ROS-damaged endoplasmic reticulum fragments are sequestered by autophagosomes, which inhibit the

release of Ca^{2+} into the cytosol and the subsequent activation of apoptosis-dependent inflammatory proteases. Activated caspase-11 is associated with IL-1b release; thus, it can activate the inflammatory response and may lead to the expansion of ischemic injury. Due to the activation of RFT (Ding et al. 2007), endoplasmic reticulum fragments can inhibit neuroinflammation and/or neuronal apoptosis, which can isolate harmful substances (Liu et al. 2010). Considering that autophagy triggered by cerebral ischemia is an endogenous neuroprotective mechanism, it is crucial that damaged proteins and/or aggregated proteins are also cleared by autophagy. Liu et al. (Liu et al. 2010) used a rat model of bilateral common carotid artery ligation and found that impaired autophagy leads to protein accumulation in related organelles (the endoplasmic reticulum, the Golgi, and mitochondria), which increases cellular stress and delays neuronal cell death.

The activity of autophagy may be related to the neuroprotective effects of ischemic preconditioning, a phenomenon in which neurons are more tolerant to ischemia due to exposure to ischemia in the past. In vitro studies have shown that an increase in autophagolysosomal synthesis and degradation after ischemic preconditioning is accompanied by an increase in the viability of PC12 cells (Park et al. 2009). Moreover, the addition of 3-MA after pretreatment with lethal oxygen-glucose deprivation (OGD) can increase the percentage of necrotic and apoptotic cells. Neuroprotection has also been observed in a mouse model of IPC after treatment with 3-MA and bafilomycin A1 (Sheng et al. 2010).

7.5.3 Autophagy Is a Metabolic Process that Delays Necrosis by Maintaining Ion Homeostasis

A key regulator of autophagy, mTOR kinase, is a sensor for changes in the intracellular ATP concentration (Adhami et al. 2007). Even a slight decrease in the cytosolic ATP concentration causes a significant increase in AMPK through the activation of adenylate kinase; thus, changes in the ATP concentration may cause changes in mTOR through the activation of AMPK. The activation of AMPK kinase can inhibit mTOR-dependent signaling pathways and induce autophagy and the inhibition of ATP-dependent metabolic pathways. It is worth mentioning that the localization of mTOR in the outer membrane and of adenylate kinase in the membrane gap can ensure that a rapid and effective response can be initiated when the intracellular AMP/ATP ratio changes (Schieke et al. 2006). However, if reperfusion does not correct the lack of energy, high levels of autophagic stress can lead to massive lysosomal activation, which leads to neuronal necrosis (Adhami et al. 2007).

7.5.4 Autophagy Participates in Ischemic Astrocyte Death

In view of the unsatisfactory clinical efficacy of many neuroprotective drugs in the past, researchers have gradually shifted their focus regarding ischemic stroke treatment to the neurovascular complex in recent years (Luo 2013). The complex is mainly composed of neurons, endothelial cells, and keratinocytes. In a series of biochemical events after ischemic stroke, astrocytes, the most abundant cells in the brain, are severely affected. Astrocytes not only protect, support, and nourish the central nervous system but also participate in water transport, metabolism, and the release of neurotransmitters, maintain the ion balance in the nervous system, synthesize neuroactive substances, regulate cerebral blood flow, and produce molecules such as neurotrophic factors (Luo 2013). It has been reported that astrocytes are adult neural progenitors in the brain and are the main source of nerve regeneration in adulthood. Astrocytes are abnormally activated after cerebral ischemia, and through the interaction between neurons and glial cells, the homeostasis of the extracellular microenvironment of brain tissue is maintained after ischemic stroke. In addition, astrocytes repair damaged neurons, promote axonal regeneration, and induce the migration of regenerating neurons (Luo 2013). In a model of permanent middle cerebral artery infarction in rats and in a model of astrocyte glucose oxygen deprivation in vitro, it has been found that autophagy, which in turn triggers the death of astrocytes, is activated in astrocytes damaged by ischemia, and that this activation of autophagy is related to the cathepsins-caspase signaling mechanism. The specific autophagy inhibitor 3-MA can effectively inhibit autophagic activity in the damaged area and prevent the autophagy-induced ischemic injury of astrocytes (Luo 2013). However, recent studies have found that autophagy caused by glial ischemic injury also contributes to ischemia-induced brain damage and neurological recovery. Autophagy is activated in damaged astrocytes, and 3-MA and BAF inhibit autophagy slightly and significantly attenuate astrocytic death during ischemic injury. A recent study observed that, in a short-term model of OGD (1 h and 4 h), the autophagy inhibitor 3-MA promotes apoptosis by increasing cleaved caspase-3; however, in a long-term model of OGD (8 and 24 h), 3-MA-treated astrocytes exhibit a significant decrease in autophagy and a time-dependent decrease in the extrinsic and intrinsic apoptotic pathways. This explains the two-way effect of autophagy in ischemic stroke.

7.5.5 Autophagy and Cerebral Ischemia/Reperfusion Injury

7.5.5.1 The Specific Role of Autophagy in Cerebral Ischemia/Reperfusion Is Still Controversial

In a rat model of middle cerebral artery ischemia/reperfusion, the activation of autophagy has been found in the cerebral ischemic penumbra, but at this time, whether the activation of autophagy is a protective mechanism or a mechanism of injury is still

unclear. Programmed cell death 5 (PDCD5) is a proapoptotic protein. In a rat model of middle cerebral artery ischemia/reperfusion, it was found that the intraventricular injection of PDCD5 siRNA can reduce PDCD5 expression, significantly improve neurobehavioral prognosis, reduce the infarct size, postinfarction cerebral edema and blood–brain barrier destruction, and reduce the expression of the autophagy marker proteins beclin1 and LC3-II/LC3-I in the penumbra. The autophagy inducer rapamycin partially attenuates the protective effect of PDCD5 siRNA, suggesting that autophagy is involved in ischemia/reperfusion brain injury (Jiang et al. 2014). However, another study found the opposite result. Hydroxysafflor yellow A is a cardioprotective drug that has been used clinically for nearly 10 years. Recent studies have found that it is beneficial in experimental cerebral infarction and acute middle cerebral artery in rats. The ischemic/reperfusion model also has a neuroprotective effect, which can significantly reduce the infarct volume of rat brain tissues exposed to middle cerebral artery ischemia/reperfusion and improve various neurological functions, activating the ischemic penumbra neurons. In the phagocytic pathway, the inhibition of the Akt-autophagy pathway by Akt inhibitors inhibits the neuroprotective effect of hydroxysafflor yellow A, suggesting that autophagy upregulation plays a neuroprotective role.

7.5.5.2 Autophagy Plays a Protective Role in Cerebral Ischemia/Reperfusion Injury by Inhibiting Inflammation

NF-κB plays an important role in initiating inflammation and ischemia/reperfusion (I/R). NF-κB is initiated after transient MCAO, and the level of NF-κB is reflected by the level of p65. w007B is a neuroprotective agent that is currently believed to be neuroprotective primarily by inhibiting inflammation, apoptosis, and autophagy. A study found that w007B significantly reduces NO levels in tissues exposed to ischemia/reperfusion (I/R) injury, reduces the p65 expression level by 82%, and inhibits the expression of the autophagy marker proteins beclin1 and LC3-II. The expression of p62 is significantly increased, suggesting that the protective effect of w007B during ischemia/reperfusion injury may be related to the inhibition of autophagic flow and a reduction in the inflammatory response induced by NF-κB (Bu et al. 2014).

7.5.6 Autophagy Inhibits Inflammatory Injury to Brain Tissue After Ischemic Stroke

A series of inflammatory reactions occur after brain tissue ischemia. Jiang et al. evaluated the mRNA and protein levels of TNFα and IL-6 and the number of activated microglia to assess the degree of inflammation in ischemic lesions. Studies

have shown that pretreatment with tetracycline can inhibit mRNA and protein levels of inflammatory TNFα after ischemic stroke, reduce the number of activated glial cells, reduce the levels of total and phosphorylated IKK, IKB, and p65, and reduce the protein expression of beclin1 and LC3. Pretreatment with the autophagy inhibitor 3-MA has similar inhibitory effects on inflammation and NF-κB activation as those of tetracycline. This suggests that tetracycline preconditioning can inhibit the inflammatory response in ischemic stroke brain tissue, which may be related to the tetracycline-mediated inhibition of autophagy, inhibition of NF-κB activation, and further inhibition of inflammatory pathway activation.

7.5.7 Autophagy and Cerebral Ischemia Adaptation

The adaptation response refers to the adaptation of normal cells to moderate environmental stress and can improve the viability of cells under stress conditions. A study found that, when brain tissue undergoes brief moderate ischemic preconditioning, the cells are more resistant to the next complete stroke. Ischemia adaptively increases the phosphorylation of AMPK, induces autophagy in the brain, and reduces the cerebral infarction volume, neurological deficits, and neuronal apoptosis. Brain slice studies have found that ischemic preconditioning can also reduce ischemia/reperfusion injury in brain tissue. The adaptive methods of cerebral ischemia, which are currently considered to have great potential for clinical application, include ischemic postconditioning and minimal ischemic preconditioning.

The mechanism of adaptive ischemia in brain tissue is unclear. Adaptive ischemia has an inhibitory effect on oxidative stress activation, mitochondrial disorders, and the activation of apoptosis. It has been reported to be related to the various processes. First, cells are activated under conditions of stress. Second messengers, such as ROS, calcium ions, 2′-deoxyuridine 5′-phosphate and sphingolipid ceramide, and some unrecognized intracellular factors that determine cell survival and death are located far from the stimulated site. Brain tissues adapt to stress after undergoing mild stimulation. Additionally, adaptive ischemia initiates a self-repair mechanism, inducing cells to produce transient responses, such as initiating autophagy and cell death mechanism, to some stressors. Finally, activated autophagy participates in repair by degrading abnormally oxidized proteins (Yu et al. 2019).

7.6 Autophagy and Atherosclerosis

Atherosclerosis is the most important cause of cerebrovascular disease. Delaying the formation of AS plaques and stabilizing them are important processes for the primary and secondary prevention of ischemic stroke. Lipid deposition in the vessel wall is an essential feature of atherosclerosis. The entire process of inflammation involved in atherosclerosis begins with inflammatory changes in endothelial cells

and is characterized by the expression of adhesion molecules. Endothelial cells are components of the inner membrane of the arterial wall and resist the adhesion of white blood cells. Foods that contain high levels of saturated fatty acids, smoking, high blood pressure, high blood sugar, obesity, and insulin resistance can lead to increased levels of adhesion molecules in endothelial cells, followed by leukocyte adhesion to the arterial wall and the endothelium under the influence of various proinflammatory chemicals. The cell layer forms atherosclerosis. In the intima of the arteries, monocytes, which are converted into macrophages that phagocytose lipids to form foam cells, also undergo inflammatory changes. T lymphocytes also migrate into the inner membrane, releasing proinflammatory cytokines to expand the inflammatory response. As these inflammatory processes progress, atherosclerosis begins and lipid lines begin to form. Inflammation is an important factor in the transformation of fatty plaques into complex plaques. Recent studies have shown that vascular endothelial cells and macrophage autophagy regulate the inflammatory response and that lipid metabolism is involved in the regulation of atherosclerosis and plaque stability.

7.6.1 Autophagy Protects Vascular Endothelial Cells by Inhibiting Inflammation

The dysfunction of endothelial cells is the starting point of atherosclerosis. Endothelial cell dysfunction caused by aging is associated with an increased risk of cerebrovascular disease in the elderly. It has been reported that the expression autophagy markers in arterial endothelial cells in the elderly is decreased by 50% and that endothelium-dependent dilatation (EDD) is reduced by 30%. Similarly, autophagy levels are reduced by 40% and EDD is reduced by 25% in the arteries of aged C57BL/6 mice. In humans and mice, damage to EDD leads to a decrease in the bioavailability of nitric oxide (NO), leading to an increase in oxidative stress and inflammation. In older mice, treatment with trehalose increases autophagy levels, which can restore NO-regulated EDD by reducing stress and general inflammatory cytokine expression. In cultured endothelial cells, the inhibition of autophagy increases oxidative stress and reduces NO production, while trehalose increases NO production through an autophagy-dependent mechanism. These results indicate that autophagy is reduced in aged vascular tissues. They show that autophagy can protect the function of the arterial endothelium by reducing oxidative stress and inflammation and increasing the bioavailability of NO.

Some of the beneficial processes in the cardiovascular system are accompanied by a reduction in inflammation. Recent studies have found that some autophagy inducers can exert vascular endothelial protection by inhibiting inflammation. In in vitro

studies, it has been shown that resveratrol can inhibit the expression of the inflammatory factor tumor necrosis factor α and adhesion molecules through autophagy-dependent pathways, thereby reducing endothelial cell dysfunction, autophagy activation and the cAMP-PRKA-AMPK-SIRT1 signaling pathways. Curcumin can induce autophagy to protect vascular endothelial cells from oxidative stress.

7.6.2 Autophagy Plays a Protective Role in Atherosclerosis by Degrading Harmful Substances

Autophagy protects atherosclerotic plaque cells from oxidative stress by degrading harmful substances, especially in the early stages before cytochrome c is released. The retention or capture of lipoproteins containing apolipoprotein B (apoB) on the arterial wall is a causative event in the pathogenesis of atherosclerosis. Autophagy degrades apoB in the late stage, and autophagy is elevated when cultured human vascular endothelial cells are exposed to oxidized low-density lipoprotein. At this time, autophagy can be promoted by inducing the degradation of oxidized low-density lipoprotein. Vascular endothelial cells play a protective role (Guan et al. 2018).

7.6.3 Autophagy in Macrophages Participates in the Process of Atherosclerosis by Regulating Inflammation

Macrophages play an important role in the immune response in atherosclerosis. Macrophage autophagy is the process by which substances in the cytoplasm are transported to lysosomes for degradation. In wild-type macrophages, autophagy regulates the activation of inflammatory bodies and limits the production of the inflammatory cytokines IL-1β and IL-18. Autophagy induced by starvation or rapamycin in human macrophages inhibits the activity of AIM2 and NLRP3 inflammatory bodies and attenuates caspase-1 activity and IL-1β secretion. Conversely, the inhibition of autophagy can increase AIM2 and NLRP3 inflammatory bodies. Corpuscle activity, as well as the autophagy adapter protein p62, has also been observed to deliver ubiquitinated inflammatory bodies to autophagosomes for degradation. After LPS stimulation, autophagy defects in macrophages caused by LC3 or beclin1 deficiency result in an increase in the activity of inflammatory bodies, leading to the secretion of active IL-1β and caspase-1 p10 subunits. Atherosclerotic plaques in macrophages lacking the major autophagy gene Atg5 are prone to apoptosis and necrosis under oxidative stress and have elevated levels of aortic IL-1β, which is associated with inflammatory corpuscle activity. These results indicate that autophagy at the basal level in macrophages can counteract apoptosis, protect macrophages from oxidative stress damage, and inhibit the inflammatory response.

The mechanisms for autophagy affecting inflammatory bodies are not well defined. One of the possible mechanisms is through the degradation of the NLRP3 protein by the autophagic lysosomal pathway, resulting in a decrease in the activity of NLRP3 inflammatory bodies. Another mechanism is through the regulation of NALP3-dependent inflammation by autophagy proteins through the preservation of the integrity of mitochondria. When autophagy is defective, damaged mitochondria and mtDNA accumulate in the cytoplasm, and mitochondria release ROS and mtDNA, which can activate inflammatory bodies, activate caspase-1, and increase the secretion of inflammatory factors. Defects in autophagy cause the long-term retention of mitochondria in the cytoplasm and the increased secretion of IL-1β, suggesting that autophagy defects affect the activity of mitochondria-mediated NLRP3 inflammatory bodies, resulting in the increased secretion of IL-1β. Another possible mechanism is through an increase in the activity of inflammatory bodies by dysfunctional lysosomes. When autophagy is defective, dysregulated lysosomal clearance is impeded by an increase in the activity of inflammatory bodies. A number of studies have shown that cathepsin B released after lysosomal injury can activate NLRP3 inflammatory bodies. Given that many organelles and cellular components are cleared by autophagy, multiple mechanisms may be involved in autophagy defects that regulate the activity of inflammatory bodies (Zhang et al. 2016).

Mild autophagy defects do not promote atherosclerosis, and only a certain degree of autophagy defects promote atherosclerosis. Consequently, do different degrees of autophagy defects have different effects on inflammation? After LPS stimulation, the level of IL-1β secreted by macrophages from Atg5 knockout mice is significantly increased compared with that in the control group, but the level of TNF-α is unchanged. However, the levels of the two cytokines secreted by beclin-1/Atg6 heterozygous macrophages are not significantly different from those in the control group. This suggests that Atg5 knockdown promotes the secretion of specific proinflammatory factors and that different degrees of autophagy deficiencies have different effects on inflammation.

In addition, macrophage autophagy is dysfunctional in more complex pathologies, and its dysregulation promotes vascular inflammation, oxidative stress, and plaque necrosis. Autophagy is impaired in the late stage of atherosclerosis, and its absence activates inflammation to promote the formation of atherosclerosis.

Factors that activate inflammatory bodies also affect autophagy. Many factors in macrophages that activate AIM2 or NLRP3 inflammatory bodies convert RalB from a GDP-bound state to a GTP-bound state, resulting in the binding of GTP-RalB to Exo84, which is the protein complex required for isolation membrane formation and maturation in autophagy. The assembly provides conditions. The induction of autophagy does not depend on apoptosis-associated speck-like protein or caspase-1 but instead on the presence of inflammatory bodies. After the activation of AIM2 inflammatory bodies, p62 is recruited, and p62 recognizes inflammatory bodies and delivers them to autophagosomes for degradation.

7.6.4 Autophagy and Atherosclerotic Plaque Stability

By observing AS plaques obtained by endometrial ablation, the number of macrophages and proinflammatory factors has been found to be significantly higher in symptomatic plaques than in asymptomatic plaques or AS plaques with a tendency to rupture. A large number of macrophage cells has been found to aggregate, and AS plaques are prone to rupture where fibrous caps are weakened, especially in the presence of a large number of macrophages. Further studies have found that macrophages can produce matrix metalloproteinases that degrade extracellular matrices, induce smooth muscle cell apoptosis, and reduce the number of collagen-supporting cells. The removal of macrophages by the macrophage-specific induction of cell death stabilizes the structure of the plaques. These results indicate that macrophages play an important role in the instability and rupture of plaques (Wang et al. 2018).

Toll-like receptors (TLRs) belong to a family of innate immune system proteins and play an important role in the early fight against pathogens. Macrophages express Toll-like receptors to recognize pathogens and to clear pathogens within cells by autophagy. TLR7 is a receptor that is expressed only in human carotid macrophages and not in vascular smooth muscle cells (VSMCs). In vitro studies have revealed that imiquimod, the ligand of TLR7, can induce the autophagic death of macrophages and that this effect is related to the activation of NF-κB, which promotes proinflammatory factors and processes related to factor release, by imiquimod. Imiquimod causes a significant decrease in cytokine release in macrophages lacking autophagy because these cells necrose at a rapid rate. A study on a rabbit model of carotid artery AS found that low concentrations of imiquimod can induce autophagy in macrophages without inducing cell death but increase the penetration of cell adhesion factor 1 and T lymphocytes by inducing cytokine production. The aggregation of macrophages expands the plaque area and promotes the progression of plaques. High concentrations of imiquimod induce the autophagic death of macrophages.

The role of macrophages in the instability of atherosclerotic plaques is more important than that in smooth muscle cells. The removal of macrophages from plaques by the selective induction of cell death in a stable artery with a tendency to rupture is an effective method for improving atherosclerotic lesions, but the mechanism by which macrophages, rather than other types of cells, such as smooth muscle cells, are specifically activated is still not well understood. Recently, it has been found that, in J774A.1 and RAW264.7 macrophages and INF-γ-sensitized murine peritoneal macrophages, the all-cysteine inhibitor Z-VAD-fmk can induce cell expression and secretion. Chemokines and cytokines, including TNFα, induce autophagy and cell necrosis but have no effect on VSMCs or C2C12 myoblasts, and the combination of Z-VAD-fmk and TNFα can also cause the necrosis of SMCs. In this regard, Z-VAD-fmk is harmful because it stimulates the inflammatory response and indirectly induces the death of SMCs. Future work is needed to study the mechanism by which macrophage plaques selectively induce nonapoptotic cell death. It is hoped that this mode of death does not activate inflammation or lead to SMC death and will

play an important role in maintaining the stability of atherosclerosis (Nabavi et al. 2019).

In contrast to basal autophagy, the excessive stimulation of autophagy can lead to the autophagic death of vascular smooth muscle cells, which in turn leads to plaque instability due to reduced collagen synthesis and the thinning of the fibrous cap. The autophagic death of endothelial cells also occurs in plaques, as endothelial damage or death represents the occurrence of acute clinical events that promote diseased thrombosis.

7.6.5 Autophagy Accelerates the Progression of Atherosclerosis by Participating in the Formation of Waxy Substances

In addition to protection, autophagy is also involved in the formation of waxy substances, insoluble protein complexes found in all human AS lesions that are associated with oxidized lipids, in atherosclerosis. Hydrogen peroxide (H_2O_2) produced by mitochondria and other organelles can penetrate into the second lysosomal cavity. These lysosomes contain iron produced by cellular structures degraded by autophagy. Through the Fenton reaction, active ferrous iron and H_2O_2 interact, leading to the production of hydroxyl groups that induce lipid peroxidation and ultimately the formation of intermolecular interactions and wax-like substances. Iron and waxy substances are codeposited in the extracellular or intracellular region of foam-like macrophages and SMCs during progressive plaque formation. In progressive plaques in humans, many cells contain a large proportion of wax-like substances that contain lysosomes, making lysosomal enzymes unable to degrade the waxy substances. These lysosomal enzymes lose their effectiveness (such as their ability to degradation substances by nascent autophagy), leading to autophagic damage and apoptosis. Impaired autophagy stimulates the further aggregation of damaged mitochondria, increases the production of reactive oxygen species, and further promotes the formation of waxy substances. Interestingly, the sustained intracellular lysosomal degradation of iron-containing substances by autophagy is associated with H_2O_2 formation and the peroxidation of lysosomal membranes, which leads to the further disintegration of lysosomes and the release of harmful lysosomal enzymes, especially under severe oxidative stress conditions. If, at a certain intensity, reparative autophagy is induced, it can cause the aggregation of iron nondegrading oxidation products, such as waxy substances. Ultimately, these events sensitize cells and cause apoptosis, and the released lysosomal enzymes can attack other proteins and mitochondria and stimulate cytochrome c release (Zhang et al. 2016).

7.7 Conclusions

In summary, a basal level of autophagy is necessary for the maintenance of the normal morphology and function of cerebral vessels. The inflammatory response is activated and autophagy levels are imbalanced in cerebral blood vessels during aging and in disease states. Under these conditions, autophagy participates in the regulation of inflammation and neuronal damage in cerebral vessels and surrounding tissues, and this regulation may be positive. It may also be negative, and this negative regulation may be related to the speed, extent, and duration of cerebrovascular disease injury and the cell types in the blood vessel wall. However, the specific role of autophagy activation in the development and progression of cerebrovascular disease, as well as when and how autophagy can regulate autophagy regulators to play a protective role in the process of cerebrovascular disease, is still unclear. New therapeutic targets must be found for the prevention and treatment of diseases.

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Chapter 8
Autophagy and Hemorrhagic Stroke

Yanlin Zhang and Chunfeng Liu

Abstract Hemorrhagic stroke includes cerebral hemorrhage and subarachnoid hemorrhage. An increasing number of studies have found that autophagy also occurs in brain tissues after cerebral hemorrhage and subarachnoid hemorrhage. The potential role of selective autophagy in the clinical treatment of hemorrhagic stroke has been recognized, but a consensus on the exact effect and function of autophagy has not been reached, and the mechanism needs to be further studied. In this chapter, the mechanism of brain injury after cerebral hemorrhage and subarachnoid hemorrhage is briefly introduced, and changes in the autophagy pathway and the role of autophagy in the process of brain injury are discussed.

Keywords Autophagy · Cerebral hemorrhage · Subarachnoid hemorrhage

8.1 Autophagy and Cerebral Hemorrhage

Intracerebral hemorrhage (ICH), also known as spontaneous cerebral hemorrhage, is the primary type of nontraumatic intracerebral hemorrhage, accounting for 20–30% of acute cerebrovascular disease, and is a type of stroke with high morbidity and mortality (Wu et al. 2010). The morbidity ranges from 60/100,000 to 80/100,000 per year, and the mortality in the acute phase is approximately 30–40%, which is the highest rate among acute cerebrovascular diseases. Only approximately 20% of ICH patients get recovery of functional independence after 6 months. The main cause of cerebral hemorrhage is cerebral arteriosclerosis induced by hypertension, aneurysm, arteriovenous malformation, amyloidosis and cavernous hemangioma (Xi et al. 2006).

The mechanism of brain injury after intracerebral hemorrhage involves primary injury that results from the extrusion of brain tissue by hematoma and the extrusion of distal brain tissue, resulting in deviation from the midline. The enlargement of the hematoma after intracerebral hemorrhage occurs in approximately one-third of patients. The enlargement of the hematoma can also affect cerebral blood flow and

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accelerate the deterioration of neurons. In addition, hematoma itself can lead to secondary brain injury that results in severe neurological impairment and sometimes delayed death (Qureshi et al. 2003). A study found that, even after blood clots are removed after surgery, delayed brain damage remains and does not improve (Xi et al. 2006). Secondary injury is caused by the damage of blood products, such as hemoglobin, inflammation caused by the stress response, ischemia and edema, to the tissues around the hematoma. Experimental studies have shown that thrombin formation, erythrocytolysis and iron toxicity play major roles in injury caused by cerebral hemorrhage. Secondary damage occurs through a complex process that can last for hours or even days. There is increasing evidence that autophagy is activated and involved in this pathophysiological process.

At present, medical treatments for cerebral hemorrhage are very limited and mainly include dehydration to reduce cranial pressure, hemostasis, antioxidative stress treatments and trophic support of nerves. To date, none of the drugs used to treat cerebral hemorrhage have been shown to extend survival in stage III clinical trials (Keep et al. 2012). Recent studies have shown that it is possible to find new therapeutic targets targeting the mechanism of injury by blood products. The challenge of autophagy-targeted therapy is to selectively induce the protective effects of autophagy without unnecessarily activating proinflammatory autophagic activity in the cell death pathway.

8.1.1 Induction of Autophagy in Brain Tissue After Cerebral Hemorrhage

Autophagy occurs in the area around the hematoma after intracerebral hemorrhage. Most autophagic brain cells are astrocytes. In a rat model, it has been found that the activation of autophagy in the brain tissue around the hemorrhagic lesion begins at 6 h, peaks at 12 h and 24 h, and decreases 72 h after cerebral hemorrhage (Chen 2012). The mechanisms of autophagy activation after cerebral hemorrhage may include: (1) iron overload and (2) thrombin accumulation.

8.1.1.1 Iron and Autophagy in Tissues After Cerebral Hemorrhage

Cerebral hemorrhage is an extreme case of iron overload in the brain. Because there are high levels of iron in the hemoglobin in hematoma, the iron concentration in the brain reaches a very high level after cerebral hemorrhage, and nonheme iron in the brain cannot be cleared within a month (Wu et al. 2003). Studies have found that the perfusion of ferric iron into the hippocampus and striatum of rats can lead to an increased conversion of the autophagy marker protein LC3-I to LC3-II, and the treatment of primary cultured neurons with iron can also lead to an increased conversion of LC3-I to LC3-II, accompanied by the accumulation of MDC-positive

substances (He et al. 2008a, b). However, deferoxamine, an iron-chelating agent, can significantly reduce autophagy-related cell death induced by cerebral hemorrhage, indicating that iron plays an important role in autophagic cell death induced by cerebral hemorrhage. In a rat model of ICH conditions, the inhibition of autophagy can reduce the severity of ferric citrate-induced striatal injury (Wu et al. 2003). According to the above studies, ferric citrate and ferrous iron induce autophagy in ICH and play a harmful role.

8.1.1.2 Thrombin and Autophagy in Cerebral Hemorrhage

Thrombin is a serine protease produced by prothrombin lysis and is an important component of the coagulation cascade. After cerebral hemorrhage, thrombin is immediately produced in the brain to induce hemostasis. However, thrombin has multiple effects in brain injury. It can kill neurons and astrocytes at high concentrations and is involved in early brain injury after cerebral hemorrhage. Related to these early effects, thrombin is also associated with cerebral resuscitation after cerebral hemorrhage (Xi et al. 2006). It has been found that thrombin can induce autophagy in the brain and cultured astrocytes. Hirudin, an inhibitor of thrombin, can reduce the level of autophagy induced by cerebral hemorrhage. 3-MA, an autophagy inhibitor, can reduce the number of MDC-labeled autophagy vesicles after thrombin treatment and increase cell death induced by thrombin (Xi et al. 2006). This suggests that thrombin is involved in the induction of autophagy after cerebral hemorrhage and that the beneficial effect of thrombin-induced autophagy is worthy of attention.

8.1.2 The Role of Autophagy in Cerebral Hemorrhage

After cerebral hemorrhage, nerve cells suffer various injuries (such as hematoma, ischemia, hypoxia, inflammatory injury, and injury due to free radicals, thrombin and other harmful substances). Noaxintong, a drug that can improve neurological functional defects after cerebral hemorrhage, is neuroprotective. It has been found to reduce the autophagy level in tissues around the hemorrhagic lesion after cerebral hemorrhage, suggesting that the inhibition of autophagy may be involved in the neuroprotective mechanism of Noaxintong under these conditions. However, more evidence is needed to confirm this hypothesis. In addition, the inhibition of autophagy also increases thrombin-induced cell death.

Autophagy is involved in the death of brain cells and inflammatory damage in patients with cerebral hemorrhage. There are three main types of cell death, namely, necrotic, apoptotic and autophagic cell death. Both necrotic cell death and apoptotic cell death occur in brain tissue after intracerebral hemorrhage (Hua et al. 2007). The excessive activation of autophagy leads to the death of neurons, so autophagy can trigger and regulate programmed cell death under specific pathological conditions (Wu et al. 2018). Recent studies have suggested that autophagic cell death occurs

after cerebral hemorrhage (Hua et al. 2007). Necroptosis is a type of programmed cell death that can be specifically inhibited by necrostatin-1. Studies on a mouse model of striatal cerebral hemorrhage have revealed that the intraventricular injection of necrostatin-1 can inhibit autophagy and apoptotic cell death in hemorrhagic mice and play a neuroprotective role (Chang et al. 2014). This suggests that necrosis, apoptosis and autophagy interact with one another.

The role of autophagy in inflammation is mediated by the unconventional secretion of cytokines or by the targeted degradation of inflammatory corpuscles (Yang et al. 2014). Microglial activation mediated by Toll-like receptor 4 (TLR4) plays an important role in inflammatory injury in cerebral hemorrhagic tissues. Studies on a mouse model of cerebral hemorrhage found that, compared with those in wild-type mice, the autophagy level and inflammatory response in Tlr4−/− mice were decreased after treatment with dissolved red blood cells. In addition, cerebral edema and nerve injury were also alleviated in Tlr4−/− mice with cerebral hemorrhage. The autophagy inhibitor 3-MA can inhibit the activation of microglial cells induced by erythrocyte lysis and inflammatory injury and improve the neurological function of rats with cerebral hemorrhage (Yang et al. 2014).

To date, the bidirectional effect of enhanced autophagy on brain injury has been confirmed, but there is evidence that the level and effect of autophagy in ICH can also depend on individual differences. Admission hyperglycemia may have adverse effects on the survival rate and functional prognosis of patients with cerebral hemorrhage. It has been found in a rat model that the autophagy level in brain tissue is increased 24 h after cerebral hemorrhage. The autophagy level in tissue surrounding cerebral hematoma in rats with hyperglycemia is lower than that in rats without hyperglycemia, and hyperglycemic rats exhibit more serious limb movement deficits and brain edema. This suggests that autophagy plays a beneficial role in cerebral hemorrhage under hyperglycemic conditions (Liu et al. 2014a; Yang et al. 2014). There is also evidence that ICH can lead to more severe autophagy and neurological dysfunction in elderly rats, suggesting the role of autophagy in brain injury in old age (Fig. 8.1).

8.1.3 Gender Dimorphism of Autophagy After Cerebral Hemorrhage

Cerebral hemorrhage has been identified as a gender dimorphic disease (Roof and Hall 2000), and compared to men, postmenopausal women exhibit better survival and functional recovery after cerebral hemorrhage (Zia et al. 2009). Estrogen has also been shown to have a protective effect in experimental cerebral hemorrhage and iron-induced brain injury (Chen et al. 2010). Studies have shown that estrogen inhibits ferrous citrate-induced autophagy through an estrogen-receptor-dependent pathway. In female rats, the inhibition of ferrous citrate-induced autophagy through an estrogen-receptor dependent pathway may be involved in the neuroprotective effect of estrogen

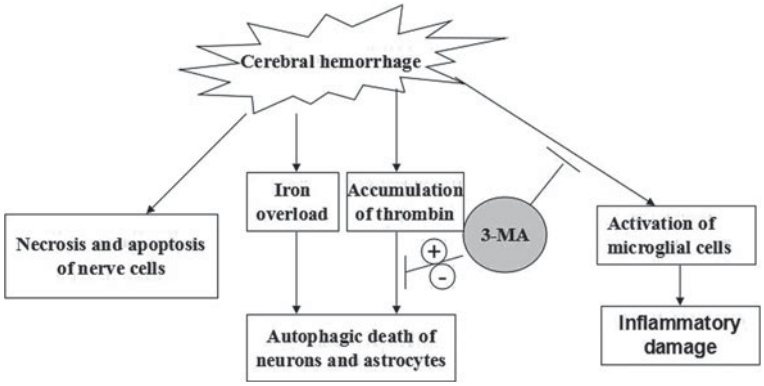

Fig. 8.1 Brain tissue injury pathway after cerebral hemorrhage. After intracerebral hemorrhage, the brain tissue surrounding the hematoma undergoes cell death characterized by necrosis, apoptosis and autophagy in nerve tissue and is associated with iron overload and thrombin accumulation. The autophagy inhibitor 3-MA can inhibit the inflammation injury caused by microglial cell activation, and it has a dual regulation effect on the autophagy induced by thrombin aggregation and apoptotic cell death

in ferrous citrate-induced brain injury. Therefore, estrogen may play a key role in the gender dimorphism of ferrous citrate-induced brain injury. Autophagic cell death may be involved in brain damage caused by iron overload, especially in men. In women with brain injury, the inhibition of ferrous citrate-induced autophagy by estrogen helps to reduce the severity of the injury. Thus, autophagy may be another factor involved in the sex dimorphism of ferrous citrate-induced brain injury. In men, there should be a stronger focus on inhibiting autophagic cell death caused by iron overload. This provides important information for the development of gender-specific treatment strategies for the prevention of cerebral dysfunction and/or iron-overload related neurodegenerative diseases caused by cerebral hemorrhage (Chen et al. 2012).

8.1.4 Age and Autophagy in Brain Tissue After Cerebral Hemorrhage

Age is an important factor affecting brain injury and prognosis in patients with cerebral hemorrhage. Cerebral hemorrhage can cause more severe brain edema and nerve function damage in old mice than in young mice (Gong et al. 2004). Normally, the autophagy level in the body shows a decreasing trend with age. However, studies have found that cerebral hemorrhage causes a stronger autophagic response in elderly rats than in young rats, combined with more severe cerebral edema and nerve function injury in elderly rats. This suggests that enhanced autophagy in elderly rats with cerebral hemorrhage may be related to more severe brain injury. In addition, elderly mice exhibit stronger microglial cell activation and more ferritin-positive cells than

young mice. Activated microglia can secrete a variety of toxic substances, and ferritin is an iron storage protein that is upregulated in the brain after cerebral hemorrhage. These results suggest that iron may be involved in autophagic induction in elderly rats. After cerebral hemorrhage in aged rats, there is a stronger autophagic response and more severe nerve function defects. Understanding the mechanism of brain injury after cerebral hemorrhage in aged rats will be helpful for developing new therapeutic strategies for hemorrhagic brain injury (Gong et al. 2011).

8.2 Autophagy and Subarachnoid Hemorrhage

Subarachnoid hemorrhage (SAH) is a devastating stroke subtype with high morbidity and mortality that accounts for 5–10% of all strokes, and most cases of SAH are caused by intracranial aneurysm rupture (Wu et al. 2010). The annual incidence of SAH caused by intracranial aneurysm rupture in China is approximately 10.5/10 million, and this type of SAH is the third most frequent acute cerebrovascular disease after cerebral ischemia and cerebral hemorrhage. Although great progress has been made in vascular interventions, surgical techniques, diagnostic methods and perioperative management, many survivors of subarachnoid hemorrhage have sustained cognitive impairment, which has an impact on functional status and quality of life. Thus, the prognosis of SAH still needs to be further improved. In recent decades, cerebral vasospasm, aneurysm rupture and rebleeding have been considered major causes of the high mortality and disability. However, a multicenter clinical trial showed that the reduction of delayed vasospasm does not improve the prognosis of patients with subarachnoid hemorrhage. Epidemiological studies have suggested that approximately two-thirds of deaths occur within 48 h after subarachnoid hemorrhage, while delayed vasospasm occurs most frequently within 4 days to 2 weeks after hemorrhage. Therefore, some scholars have proposed that early brain injury is the main cause of death and disability after subarachnoid hemorrhage (Lee et al. 2009).

Early brain injury (EBI), which plays a decisive role in the prognosis of patients, refers to brain injury that occurs between the start of subarachnoid hemorrhage to 72 h later (Jing 2012). At present, the pathophysiological mechanisms of early brain injury after subarachnoid hemorrhage, including increased intracranial pressure, decreased cerebral blood flow, decreased cerebral perfusion pressure, the inhibition of aerobic respiration, the destruction of the blood–brain barrier, cerebral edema, inflammation, oxidative stress and the death of neurons, are preliminarily understood (Yan et al. 2014). Studies have shown that the autophagic lysosomal system is involved in early and late brain injury after SAH, and new therapeutic strategy for SAH involving the regulation of autophagy has been developed (Li et al. 2017). However, more studies are needed on the exact mechanism, especially the interaction between autophagy and dysfunctional subcellular organelles (Chen et al. 2015).

8.2.1 Activation of Autophagy in Brain Tissue After Subarachnoid Hemorrhage

The direct consequence of blood–brain barrier dysfunction after subarachnoid hemorrhage is cerebral edema, which is the main component of early brain injury. A study found that the water content in both hemispheres increases within 24 h after subarachnoid hemorrhage but that the change in edema is more obvious in the ipsilateral hemisphere.

After subarachnoid hemorrhage, autophagy increases significantly in the ipsilateral frontal cortical region and persists throughout the early stages of brain injury (3 days). Previous studies have shown that, within 24 h after subarachnoid hemorrhage, the levels of the autophagy marker proteins LC3-II and Beclin-1 rapidly increase in brain tissues and peak. At the same time, the content of the lysosomal enzyme cathepsin-d also increases immediately and reaches a peak within 24 h, and the inhibition of this enzyme reduces the upregulation of LC3-II in cerebral ischemia and inhibits the formation of autophagic vesicles, which is complicated by the upregulation of LC3-II. This suggests that the early activation of autophagic circulation occurs in subarachnoid hemorrhage. Beclin-1, a protein that interacts with Bcl-2, is a component of the phosphatidylinositol 3-kinase complex. It is required for autophagy and is a marker of autophagic activation. The neuronal localization of this marker has been found; Beclin-1-positive cells are more obvious in the ipsilateral frontal part of the cerebral cortex, while there are many apoptotic cells in the superficial layer. However, in other studies, Beclin-1-positive cells induced by cerebral ischemia and hypoxia have been found in the superficial layer (Lee et al. 2009). This indicates that the autophagic activity in the ipsilateral frontal lobe is significantly increased in the early stage of brain injury after subarachnoid hemorrhage. After subarachnoid hemorrhage, ischemia and hypoxia, the localization of autophagic cells may indicate different injury mechanisms and different roles of autophagy. More experiments are needed to elucidate the molecular and biochemical mechanisms of autophagy and determine whether autophagy is protective or harmful after subarachnoid hemorrhage.

8.2.2 The Protective Effect of Autophagy in Subarachnoid Hemorrhage

Autophagy is activated in the brains of rats after subarachnoid hemorrhage in the early stage, and the autophagic activity is found to be strongest 24 h after hemorrhage and recovers 48 h after hemorrhage (Wang et al. 2012). Earlier studies have shown that a SAH-induced increase in the LC3 and Beclin-1 levels is regulated by the intraventricular administration of rapamycin and 3-MA. The autophagy activator rapamycin can improve cerebral edema, BBB permeability and clinical behavioral function. Parameters related to early brain injury are significantly aggravated after

treatment with the autophagy inhibitor 3-MA (Ji and Chen 2016). In recent years, a series of studies have found that multiple drugs may play a protective neuroprotective role in early SAH by inducing autophagy (Wang et al. 2012). Simvastatin pretreatment can enhance autophagy and inhibit neuronal apoptosis. The intracisternal injection of cystatin C enhances autophagy in the basilar artery wall 48 h after SAH. Melatonin can enhance autophagy and improve apoptotic cell death in SAH rats. TSA-enhanced autophagy can also reduce neuronal apoptosis and improve neural function. tHBQ plays a neuroprotective role in EBI by enhancing autophagy (Li et al. 2015). These findings suggest that the activation of the autophagy pathway has potential protective effects against early brain injury in a rat model of SAH model and that the protective effects are mainly reflected by the five factors described below.

8.2.2.1 Endoplasmic Reticulum Stress and Autophagy in Brain Tissue After Subarachnoid Hemorrhage

The exact mechanism of early brain injury after SAH remains unclear, and apoptosis has been reported to be an important factor in this mechanism. The endoplasmic reticulum (ER) is a necessary site for cells to synthesize and fold secreted and membrane proteins. The aggregation of misfolded proteins in the ER lumen induces ER stress (Chen et al. 2016). To maintain a stable intracellular environment, ER stress triggers a series of intracellular responses. The ability of the endoplasmic reticulum to respond to stress is necessary for cell survival. Studies (Yan et al. 2014; Chen et al. 2016) have shown that endoplasmic reticulum stress is activated 24 h after SAH in rat models. The enhanced endoplasmic reticulum stress has a neuroprotective effect, and autophagy is the downstream pathway of the protective effect induced by endoplasmic reticulum stress. The activation of endoplasmic reticulum stress can improve deficits in neuronal function in SAH mice, reduce the expression of caspase-3, a proapoptotic molecule, reduce apoptosis, reduce the number of TUNEL-positive cells and increase autophagic activity. The endoplasmic reticulum stress inducer Tm promotes autophagy and upregulates the autophagy proteins Beclin-1 and LC3-II, while the endoplasmic reticulum inhibitor TUDCA reduces autophagy activation. The inhibition of autophagy by 3-MA can inhibit the protective effect induced by endoplasmic reticulum stress. In other words, increased ER stress can protect against cell death in a mouse model of SAH through activating autophagy, inhibiting apoptosis and improving early brain injury after SAH (Yan et al. 2014).

8.2.2.2 Subarachnoid Hemorrhage and Mitochondrial Autophagy

Mitochondrial autophagy is a term used to describe the selective removal of mitochondria by autophagy. Early on, it refers to the maintenance of healthy mitochondria and the selective removal of dysfunctional mitochondria. It has been confirmed that the function of mitochondria in cortical neurons is impaired in EBI after SAH recently. SB203580 and tea polyphenols can prevent mitochondrial depolarization,

increase the ATP content and reduce the release of cytochrome c. After the administration of these substances, nerve cell apoptosis induced by mitochondrial injury is inhibited. Studies have shown that mitochondrial dysfunction plays an important role in the pathogenesis of SAH, especially in apoptosis. When the voltage-dependent anion channel (VDAC) is inhibited by VDAC1 siRNA, the expression of LC3 is reduced, but ROS protein accumulation, apoptosis and necrosis are upregulated. The level of the caspase-3 protein is also increased. However, when autophagy is activated by rapamycin, the expression of LC3 is significantly increased, while the production of ROS is decreased. Apoptosis and necrosis are prevented, and the level of the caspase-3 protein is reduced. This suggests that autophagy induced by VDAC may be mitochondrial autophagy, which may represent a therapeutic target for maintaining neuronal activity and preventing cell death in the future. This study proves that, after subarachnoid hemorrhage injury, mitochondrial autophagy is stimulated through the VDAC pathway and that VDAC promotes mitochondrial autophagy to protect nerve tissue from early apoptosis and necrosis after subarachnoid hemorrhage (Li et al. 2014).

8.2.2.3 Autophagy and Apoptosis in Brain Tissue After Subarachnoid Hemorrhage

The mechanisms of cell death after subarachnoid hemorrhage include apoptosis and necrosis. Recent studies have found that the processes of autophagy, apoptosis and necrosis interact with one another (Fang et al. 2018). After subarachnoid hemorrhage, apoptotic nerve cell death is activated by increased intracranial pressure, decreased cerebral perfusion pressure, transient global cerebral ischemia, the toxicity of subarachnoid blood clots and oxidative stress products, and leads to damages of the blood–brain barrier and cerebral edema. The inhibition of apoptosis has been proven to have a neuroprotective effect after subarachnoid hemorrhage and has become an important target for the prevention and treatment of early brain injury after subarachnoid hemorrhage. Autophagy is activated in neurons in the acute phase after SAH, although the role of autophagy in early brain injury after subarachnoid hemorrhage remains controversial. Whether autophagy activation can cause cell death or prevent cell death as part of endogenous neuroprotective effects remains to be determined. However, previous studies have shown that autophagy occurs within 6 h after subarachnoid hemorrhage, and the activation of autophagy is accompanied by the inhibition of apoptosis and improvement of early brain injury. In a model of subarachnoid hemorrhage, rapamycin treatment can induce the formation of neuronal autophagic vesicles, while 3-MA can induce neuronal apoptosis. Rapamycin treatment can significantly increase the expression of Atg5, Beclin-1 and LC3-II, reduce the activity of the apoptotic protein caspase-3, reduce the number of TUNEL-positive cells, and improve early cerebral edema and deficits in neuronal function after subarachnoid hemorrhage. In contrast, 3-MA treatment results in opposite changes in autophagy and aggravates early brain injury. Rapamycin treatment reduces the transport of Bax to mitochondria and the release of cytochrome c from mitochondria to the

cytoplasm. It has been suggested that that activation of autophagy can reduce early brain injury after subarachnoid hemorrhage. This neuroprotective effect is exerted through an antiapoptosis mechanism, and this antiapoptotic effect is related to the mitochondrial pathway (Jing et al. 2012). After subarachnoid hemorrhage, the preventive use of simvastatin can activate autophagy, inhibit apoptosis and improve early brain injury. Mortality is also lower in animals with subarachnoid hemorrhage treated with rapamycin and simvastatin compared with that in the control group. These results also suggest that autophagy activation after subarachnoid hemorrhage plays a neuroprotective role by inhibiting apoptosis (Zhao et al. 2013).

Recently, melatonin has been reported as a regulator of autophagy, and studies have proven that melatonin is a powerful antioxidant that has a beneficial effect on EBI after SAH in rats and can reduce mortality and the water content in the brain (Chen et al. 2014). Melatonin can protect against brain injury caused by ischemia/reperfusion by inducing autophagy. The increased transport of Bax to mitochondria and release of cytochrome c into the cytoplasm induced by subarachnoid hemorrhage indicate that the mitochondrial apoptosis pathway is activated in a model of subarachnoid hemorrhage. These changes result in the activation of downstream caspases, including cleaved caspase-3, leading to the apoptosis of nerve cells. Treatment with melatonin after subarachnoid hemorrhage can reduce caspase-3 activity and the number of TUNEL-positive cells and upregulate the expression of the LC3-II protein in brain tissue. In this stage, melatonin-induced autophagy can reduce the death of apoptotic cells by inhibiting the transport of Bax to mitochondria and the release of cytochrome c into the cytoplasm. These findings support that the application of melatonin after subarachnoid hemorrhage can activate the death of antiapoptotic cells through autophagy and that melatonin can activate autophagy and play a neuroprotective role by inhibiting apoptosis in the mitochondrial pathway (Chen et al. 2014).

8.2.2.4 Autophagy and Cerebral Edema After Subarachnoid Hemorrhage

Serum cystatin C (CysC) is considered as an important endogenous inhibitor of cysteine protease activity; its function in the brain is not yet clear, but it has been shown to delay neuronal degeneration and repair the nervous system (Urday et al. 2015). CysC is a candidate therapeutic agent that potentially prevents brain injury and neurodegenerative disease. A study found an increase in the water content in both hemispheres 48 h after subarachnoid hemorrhage. Treatment with low and intermediate doses of CysC significantly reduces edema formation, protects the BBB and improves early brain injury, including neurologic test scores, cerebral edema and BBB injury, after subarachnoid hemorrhage. Autophagy also increases significantly after SAH, and the autophagy level increases further after CysC administration, suggesting that the neuroprotective effect of CysC after SAH may be related to its upregulation of autophagy (Liu et al. 2014b). However, CysC metabolism and signaling pathways

related to CysC throughout the brain after subarachnoid hemorrhage remain unclear and require further study.

8.2.2.5 Autophagy and Cerebral Vasospasm After Subarachnoid Hemorrhage

Cerebral vasospasm is a common and serious complication of subarachnoid hemorrhage that occurs in the cerebral cistern, and it is an important cause of morbidity and mortality in neurologic patients. A study found that the expression of the autophagy-related proteins LC3 and Beclin-1 is low in normal controls. The expression of autophagy proteins in the basilar artery wall increases in the early stage of hemorrhage in SAH rats; the expression of autophagy proteins in the basilar artery wall increases significantly 48 h after SAH, and this effect can be enhanced by CysC treatment. In addition, compared with that in controls, the degree of cerebral vasospasm in SAH rats treated with CysC is significantly improved (Liu et al. 2013) and is consistent with the degree of autophagy in the basilar artery wall induced by CysC. These results suggest that SAH may induce autophagy in spasmodic vessels and play a role in the pathogenesis of vasospasm. The beneficial therapeutic effect of CysC treatment in SAH may be due to its beneficial regulatory effect on the autophagy signaling pathway, suggesting that autophagy activation may have a protective effect on the formation of cerebral vasospasm after SAH (Fig. 8.2).

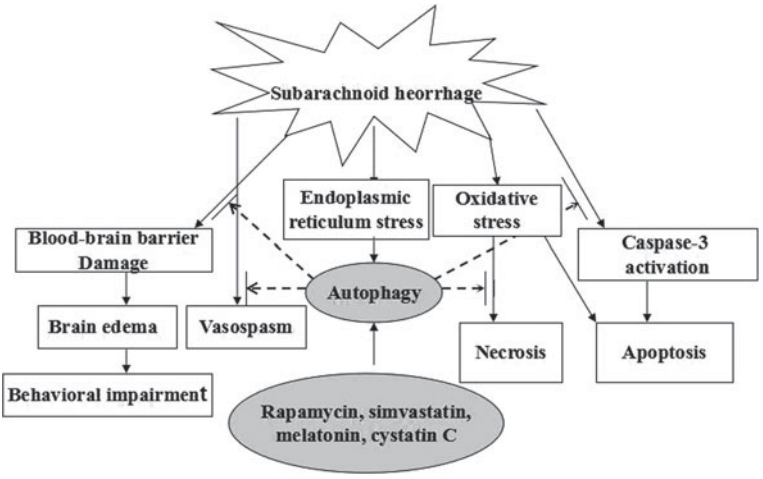

Fig. 8.2 Early brain injury pathway after subarachnoid hemorrhage. In the early stage of subarachnoid hemorrhage, brain tissue damage including cerebral edema, nerve cell necrosis, apoptosis and vasospasm occur. In this stage, autophagy activated mainly by endoplasmic reticulum stress can play a neuroprotective role and improve behavioral function by inhibiting the above damaged pathways. Simvatatin, melatonin and Cystatin-c can inhibit apoptosis by activating autophagy and play a neuroprotective role in early SAH

8.3 Conclusions

To date, studies on autophagy in hemorrhagic cerebral apoplexy have been relatively limited. Only a few studies have suggested that autophagy is involved in bleeding, early neuronal cell death and inflammation damage in cerebral hemorrhage. Inhibiting autophagy can increase brain damage caused by thrombin and hyperglycemia. There is no evidence that the induction of autophagy plays a protective role against acute cerebral hemorrhage. In subarachnoid hemorrhage, early autophagy induction may play a protective role in the brain by inhibiting cerebral edema, neuronal cell apoptosis, necrosis and cerebral vasospasm.

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Chapter 9
Autophagy and Polyglutamine Disease

Haigang Ren, Zongbing Hao, and Guanghui Wang

Abstract Polyglutamine (polyQ) disease is a type of fatal neurodegenerative disease caused by an expansion of CAG repeats in a specific gene, resulting in a protein with an abnormal polyQ fragment. The age of onset and the degree of pathological deterioration are related to the length of the polyQ fragment. At least 9 kinds of polyglutamine diseases have been discovered, including Huntington disease (HD), dentatorubral pallidoluysian atrophy (DRPLA), spinobulbar muscular atrophy (SBMA) and six spinocerebellar ataxia (SCA) such as SCA1, 2, 3, 6, 7 and 17 subtypes (Table 9.1). Previous studies suggest that autophagy plays a major role in the quality control of disease proteins in polyQ diseases. In this chapter, we majorly focused on three representative polyQ diseases, including spinocerebellar Ataxia type 3 (SCA3), spinocerebellar ataxia type 7 (SCA7) and Huntington's disease (HD). The relationship of the ubiquitin-proteasome system and autophagy involved in disease protein accumulation were summarized.

Keywords Polyglutamine disease · SCA3 · SCA7 · Huntington's disease · Autophagy

9.1 Introduction

The pathogenesis of these polyQ diseases is currently unclear. Previous studies have shown that protein aggregation, cell homeostasis, axonal transport, vesicle transport, neurotoxicity, gene transcriptional change, mitochondrial damage, and protein degradation abnormality participate in the onset and progression of the disease. Although each pathogenic protein has its own unique protein function, the numbers of CAG repeats is similar (more than 40 replicates) during the onset of different polyQ diseases. Scientists speculate that these diseases share similar pathogenesis. An important feature of the protein is that the abnormal expansion of the encoded glutamine

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can cause changes in the structure of the protein, making it easier to form aggregates, and the degradation of aggregated proteins is closely related to the proteasome or autophagy pathway. It is worth noting that long polyQ fragment proteins are difficult to degraded by proteasomes, and both aggregated and soluble forms are more prone to degradation by autophagy pathways.

Here, we introduce the characteristics of polyglutamine diseases and the relationship between autophagy and several types of spinocerebellar degeneration and Huntington's disease (Table 9.1).

9.2 Spinocerebellar Ataxia Type 3

Spinocerebellar Ataxia type 3 (SCA3), also known as Machado-Joseph Disease (MJD), is an autosomal dominant late-onset neurodegenerative disease and a clinically common type of spinal cerebellar degeneration disease. SCA3 reported in foreign countries accounts for 40–46% of hereditary ataxia, and accounts for about 48% in China. SCA3 is more common in countries such as China, Germany, and Portugal. The main symptoms are cerebellar ataxia, pyramidal and extrapyramidal symptoms, exophthalmos, progressive extraocular muscle paralysis, and facial convulsions.

SCA3 is caused by the increased number of CAG repeats near the 3′ end of the *MJD1* gene, and the protein encoded by the *MJD1* gene is called ataxin-3. The normal human *MJD1* gene has a CAG expansion of 14–36 repeats, while the expansions reached 52–86 repeats in patients. The *MJD1* gene was cloned in 1994 and is located on the long arm of chromosome 14 and contains 11 exons. The *MJD1* mRNA is widely expressed in a plurality of tissues and has different homologues because of alternative splicing.

It has a conserved sequence at the 5′ end of the *MJD1* gene, encoding a Josephin domain with ubiquitin hydrolase activity. The middle segment of the encoded protein has two conserved ubiquitin interacting motifs (UIMs), which bind to the polyubiquitin chain and has a CAG repeat at the 3′ end. The repeats of CAG expansion closely related to the age of onset and the severity of the patients. The more number of repeats, the earlier the onset of the disease, and the more serious the clinical symptoms. Therefore, the abnormal expansions of CAG repeat can be used as the basis for genetic diagnosis. The structure of the protein is unstable due to the expanded polyQ, and as the number of polyQ increases, the ataxin-3 protein is more likely to form a poorly soluble β-sheet structure. Studies have confirmed that there are a large number of inclusion bodies formed by the abnormal accumulation of ataxin-3 in the brain of SCA3 patients, and such aggregates are particularly common in most severe areas of neuronal damage. Transfection of the *MJD1* gene containing a long CAG repeat in cultured cells caused aggregation of the ataxin-3 protein and cell death. Therefore, it has long been considered that aggregation of ataxin-3 is "gain of function". However, more and more studies recently showed that protein aggregation may not be a direct

Table 9.1 Classification of poly-glutamine disease

Polyglutamine disease	Location	Genes	Protein	Clinical symptoms
Spinobulbar muscular atrophy	Xq13–q21	AR	Androgen receptor	Amyosthenia, motor dysfuntion
Huntington's disease	4p16.3	IT15	Huntingtin	Chorea, dementia
Dentatorubral pallidoluysian atrophy	12p13.31	DRPLA	Atrophin 1	Myoclonic epilepsy, ataxia, dementia
Spinocerebellar Ataxia type 1	6p23	SCA1	Ataxin 1	Ataxia, dementia, amyotrophy, peripheral neuropathy
Spinocerebellar Ataxia type 2	12q24.1	SCA2	Ataxin 2	Progressive ataxia, dysarthria and dysphagia, ocular motility disorders, cerebellar action and postural tremor, autonomic dysfunction, cognition
Spinocerebellar Ataxia type 3	14q32.1	SCA3/MJD	Ataxin 3	Dysarthria and eyelid retraction, ocular motility disorders, dystonia
Spinocerebellar Ataxia type 6	19p13	CACNA1A	α_{1A}-voltage-dependent calcium channel subunit	Dysarthria, intentional tremor, dysphagia with sensory, pyramidal and extrapyramidal motor dysfunction
Spinocerebellar Ataxia type 7	3p12–p13	SCA7	Ataxin 7	Slower saccades, ophthalmoplegia, acataposis, somatic and neuropsychological deficit, vision and retinal cone photoreceptor cell deficits
Spinocerebellar Ataxia type 17	6q27	TBP	TATA box binding protein	Ataxia, dystonia and psychotic symptoms

cause of the disease, but a self-protection effect of late-onset disease. Misfolded protein was recruited to the aggregates to protect cell from damage in stress. Therefore, as with the aggregation of other neurodegenerative diseases, whether the aggregates of ataxin-3 are cytotoxic or self-protection remained unknown.

9.2.1 The Relationship Between SCA3 and Ubiquitin-Proteasome System

The ataxin-3 protein binds to the ubiquitinated protein through the UIM domain and acts as an ubiquitin hydrolase. On the other hand, if not degraded in time, the misfolded polyQ in the carboxy-terminal will form an aggregation and that is cytotoxicity. It can be seen that the ataxin-3 protein not only mediates the ubiquitin chain editing of the ubiquitinated protein but also becomes a substrate for the ubiquitin-proteasome degradation system after the abnormal expansion of polyQ fragment.

Poly-ubiquitination has multiple functions, and the ubiquitin chains of ubiquitin K48 and K63 linkages have been extensively studied. It is generally believed that K48-linked poly-ubiquitin chain marked the degradation of substrate protein through the proteasome, and the poly-ubiquitination of the K63 linkage mediated DNA repair, ribosome function, and formation of aggresome. Ataxin-3 binds to ubiquitinated proteins through the UIM structure, especially the protein that link four or more ubiquitin chains. Ataxin-3 was first discovered to digest the K48 poly-ubiquitinated protein and inhibit the degradation of substrate proteins, while some researchers recently believe that the length of ubiquitin chain can be properly edited by ataxin-3 to promote the degradation of the substrate through the proteasome.

9.2.2 The Role of Autophagy in the Pathogenesis of SCA3

Autophagy acts as an important pathway for the degragation of long-lived proteins, protein aggregates and damaged organelles in cells, and plays a major role in many neurodegenerative diseases. In the *Drosophila* model, genetic inhibition of autophagy can significantly aggravate the neurotoxicity of polyQ-ataxin-3 on retinal cells, suggesting the protective role of autophagy in SCA3. The Rapamycin, which is an inducer of autophagy, can enhance the activity of autophagy and promote the degradation of ataxin-3 aggregation by inhibiting mTOR. In the cell line of ataxin-3 overexpression with 70Q repeat expansions, the Rapamycin accelerated the clearance of the mutant ataxin-3 protein, while Bafilomycin A1 (Baf A1), the inhibitor of autophagy, treatment reduced the degradation of the mutant ataxin-3 protein. And the derivative of Rapamycin, temsirolimus (CCI-779), reduced the protein levels

of mutated ataxin-3 and the formation of aggregations, accompanied with ameliorated motor function of *MJD1*-70Q transgenic mice. Interestingly, CCI-779 had no effect on the expression of endogenous ataxin-3, suggesting that CCI-779 can be a long-term treatment for SCA3 (Menzies et al. 2010).

The expression of endogenous autophagy markers such as p62, Atg16L and punctate LC3 was abnormal in the putamen of SCA3 patients and similar neuropathologies were also appeared in the brain of 71Q-ataxin-3 transgenic mice. The aggregations of autophagosome can also been detected in the brain of late stage polyQ-ataxin-3 lentivirus infected mice. The expression of Beclin 1 was significantly downregulated in the striatum of polyQ-ataxin-3 mice and brain tissues of SCA3 patients compared to controls (Nascimento-Ferreira et al. 2011). Beclin 1 is an important protein in the process of autophagosome formation, which can mediate the localization of other autophagic factors in phagocytic vesicles, and regulate the formation and maturation of autophagosomes. Overexpression of Beclin 1 in the brain of SCA3 rats promoted the increase of autophagy and reduced the ubiquitin positive aggregates of mutated ataxin-3 in the cytoplasm and nucleus, and decreased cell death caused by ataxin-3 aggregates. Ten weeks after infection with Beclin 1 lentivirus in the cerebellum of 3 weeks old MJD1-CAG72 transgenic mice, the motor coordination, stride strength and stride width were significantly improved compared with control transgenic mice, and the disease progression of transgenic mice were significantly slowed. The Purkinje cells play an essential role in the motor coordination and the deficiency of Purkinje cells may cause ataxia. Decreased processes of dendrites and expression of DARPP-32 were found in cerebellum of MJD1-CAG72 transgenic mice, while the overexpression of Beclin 1 reduced the aggregates of polyQ and maintained the dendritic morphology (Nascimento-Ferreira et al. 2013).

In addition, autophagy can be promoted by the administration of HSP90 inhibitor 17-Dimethylaminoethylamino-17-demethoxygeldanamycin (17-DAMG), via reducing the formation of 135Q-ataxin-3 and increasing the expressions of Beclin 1 and LC3-II.

Previous study found that Puromycin-sensitive aminopeptidase (PSA) protected transgenic Drosophila or mice with long repeat polyQ. Inhibition of PSA significantly increased protein levels of ataxin-3 pathological mutations, while overexpression of PSA significantly inhibited the aggregation and cytotoxicity. Further experiments showed that overexpression of PSA significantly upregulated the level of LC3-II, while silencing or inhibiting the activity of PSA significantly downregulated the levels of LC3-II, suggesting that PSA removed the misfolded proteins with aggregation tendency by increasing the level of autophagy, thereby protecting cells from toxic damage caused by the aggregations.

In summary, the polyQ expansion in the ataxin-3 is a substrate for autophagy. Mutated ataxin-3 is degraded by the autophagy pathway either through drug-induced autophagy or overexpression of major signaling factor in autophagy. Therefore, enhanced autophagy is thought as a potential target for spinocerebellar ataxia diseases including SCA3.

9.2.3 *The Regulation of Ataxin-3 on Autophagy*

The ubiquitination of proteins plays an important role in the regulation of autophagy. As an ubiquitin hydrolase, ataxin-3 participates in the autophagy degradation pathway through regulating the ubiquitin chain of substrates. Parkinson's disease-related protein parkin is an E3 ligase that plays an important role in the regulation of mitophagy. Ataxin-3 binds parkin or polyubiquitinated parkin through multiple domains, reducing the level of ubiquitination of parkin. Interestingly, mutant ataxin-3 has higher ubiquitin hydrolase activity than wild-type ataxin-3 and is more efficient at clearing K27 and K29 linked polyubiquitinated parkin. It is worth noting that both wild-type and mutant ataxin-3 can hydrolyze the ubiquitin chain of parkin, and the mutant ataxin-3 can promote the degradation of parkin through autophagy, thus lower expression of parkin in wild-type *MJD1* and mutant *MJD1* transgenic mice compared to controls. Mutant ataxin-3 impaired the ubiquitination of parkin and mitophagy, and mitochondrial abnormalities were determined in polyQ disease models including SCA3 (Durcan and Fon 2011; Durcan et al. 2011, 2012). Ataxin-3 mediates the aggregation of the substrate protein SOD1 through the editing of the K63-linked polyubiquitin chain, which eventually degrades through the autophagy pathway, thereby acting as a cytoprotective effect. In 2014, Zhou et al. showed that the autophagy adaptor protein p62 can directly bind to ataxin-3 and regulate the aggregation of ataxin-3 pathological mutants in cells, thereby protecting cells from the damage of mutant ataxin-3 (Zhou et al. 2014).

The balance between ubiquitin-proteasome and autophagy is particularly important for the cellular homeostasis. Ataxin-3 binds to key factors in the ubiquitin proteasome and autophagy pathway, act as a "hairdresser", modifies the ubiquitin of substrate protein to maintain the balance of cellular quality control and protein homeostasis. For example, a study reported that wild-type ataxin-3 interacts with beclin 1, which allows deubiquitinase activity of ataxin-3 to protect beclin 1 from ubiquitin proteasome-mediated degradation thereby promotes autophagy. Whereas mutant polyQ expansion ataxin-3 competes with wild-type ataxin-3 that binds to beclin 1, thereby inhibits the expression of beclin 1 and autophagy (Ashkenazi et al. 2017). Ataxin-3, same as p62, acts as a major balancer between ubiquitin proteasome system and autophagy pathway.

9.3 Autophagy and Spinocerebellar Ataxia Type 7

Spinocerebellar ataxia type 7 (SCA7), is also a progressive autosomal dominant hereditary spinocerebellar ataxia. It is commonly happened in European and American but rare in China. The disease is mainly caused by damage to neurons in the cerebellum and retina, with pathological features of cerebellar ataxia, visual impairment, retinal degeneration and atrophy of the cerebellum and brainstem.

The causative gene of SCA7 is located on chromosome 3, and the CAG repeat is located in the first exon at the 5′ end of the gene. The gene encoding protein is ataxin-7 and contains 892 amino acids. The normal ataxin-7 protein is evenly distributed throughout the whole cell and is a component of two types of transcription factor complexes, TFTC and STAGA. There is also evidence that ataxin-7 may be involved in the regulation of cytoskeletal stability. The ataxin-7 gene in normal humans contains 4–35 CAG repeats, while extends to 36–300 in SCA7 patients, resulting in the aggregation of the mutant ataxin-7 with cytotoxicity. On the other hand, the long repeat length of polyQ also leads to ataxin-7 loss of function. In SCA7, the ataxin-7 can be cleaved by caspase-7, resulting fragments containing polyQ repeat expansion in the amino terminal. And aggregates of the fragments were found in brain of SCA7 patients, which is similar to other polyQ disease. Due to the close relationship between misfolded protein, abnormal aggregation and intracellular protein degradation systems, it has been concerned whether ataxin-7 can be degraded by the ubiquitin proteasome or autophagy.

Co-localization of ataxin-7 pathological fragments and autophagic markers was found in cultured cells, and aggregation of pathological fragments increased lysosomal-like subcellular structure in cultured cells and primary cortical neurons. The wild-type ataxin-7 is only degraded by autophagy, while the mutant ataxin-7 can be degraded through either proteasome system or autophagy pathway. The results of immunohistochemistry showed that ataxin-7 co-distributed with LAMP-2A, a marker of chaperone-mediated autophagy, and the expression of LC-3II significantly increased in SCA7 transgenic mice. The interaction between Tp53 and FIP200 was enhanced and recruited to the aggregates of ataxin-7, thereby reducing the soluble FIP200 and causing the instability of ULK1, which is an initiator of autophagy. The inhibitor of Tp53 or ataxin-7 aggregates restored the expression of ULK1 and soluble FIP200 thereby enables autophagy and ameliorated the toxicity of ataxin-7.

The ataxin-7 aggregates in the brain of ataxin-7 knock-in mice and co-localizes with autophagy-related proteins such as mTOR, beclin 1, p62 and ubiquitin (Ub). Large number of inclusions contains autophagosomes and autophagy-related proteins, such as LC3, LAMP-1, LAMP-2 and Cathepsin-D, were determined in the cerebellum of ataxin-7 knock-in mice. In addition, abnormal aggregations of autophagic markers can also be seen in SCA7 autopsy brain and peripheral mononuclear blood cells of patients. Since ataxin-7 specifically aggregates in the cerebellum, which may be responsible for the accumulation of autophagic markers thereby leads to a decrease in the function of autophagy-lysosomes. Consistently, the autophagic flow was blocked in the mutant ataxin-7 transfected cells, and the expression of autophagy-related protein Atg12 in the peripheral mononuclear cells of the patients also increased. Therefore, there is a strong interaction between ataxin-7 and autophagy, which is likely to be involved in the development of SCA7 disease. In SCA7 disease, ataxin-7 aggregates increased the expression of autophagy-related markers, accompanied with declined autophagic function. On the other hand, autophagy regulates the degradation of ataxin-7 aggregates, which can be a potential treatment for SCA7.

9.4 Autophagy and Huntington's Disease

Huntington's disease (HD), also known as hereditary chorea, is clinically characterized as wide involuntary movement of limbs, accompanied by cognitive impairment and mental disorders. The lesion involves mainly in the striatum and cerebral cortex. The main pathological changes of HD are premature death of striatum-projecting γ-aminobutyric acid (GABA) neurons and the pyramidal cells of cerebral motor cortex, atrophy of the cerebral cortex, and enlargement of the ventricular system. A large number of inclusion bodies formed by protein aggregation are found in the nucleus of neurons in the brain of HD patients, which is a landmark neuropathological feature. HD is a progressive disease and is difficult to diagnose at the beginning. Typical symptoms usually occur between the ages of 35–44 and disease progression is about 15–20 years. Familial inheritance may occur in childhood with a shorter disease progression, about 7–10 years.

Interesting transcript 15 (IT15), as the pathogenic gene of HD, consists of 67 exons and encodes a 3144 amino acid protein with a relative molecular weight of 348 kDa, named Huntingtin (Htt). HD is also a polyglutamine disease with a CAG-encoded polyQ, starting from the 17th amino acid residue at the N terminus of Htt gene and presenting polymorphic. The repetition of this sequence is related to age of onset, the more repeats, the earlier the age of onset comes. The length of CAG is shorter than 35 repeats in the normal population, generally between 16–26, while HD patients with more than 37 repeats, patients carries 40–50 repeats begins in middle age. While the length over 100 repeats, adolescence instead.

When the polyQ sequence contained in Htt is prolonged, Htt cross-links under the reaction of transglutaminase, which promotes the transformation from α-helix to β-sheet. The structural conversion of the α-helix/β-sheet results in the exposure of the hydrophobic group and the hydrophilic group is buried inside the protein, causing a cross-β-sheet structure between molecules. When the length of polyQ exceeds 37 repeats, the β-sheet structure formed by Htt is relatively stable. This β-sheet-based protein is prone to form dimers and multimers. Due to the exposure of hydrophobic groups, these aggregates are difficult to dissolve in hydrophellolic environment, finally aggregate in cytoplasm or forms intranuclear inclusions in nucleus. The unusual increase will cause metabolism system disorder, thereby breaking the homeostasis, causing loss of normal cell functions, and leading to cell death.

9.4.1 The Correlation Between Ubiquitin-Proteasome Pathway and Huntingtin Protein Metabolism

Hayden's team found that Htt interacts with the ubiquitin-coupled enzyme E2-25 K, and E2-25 K is involved in the ubiquitination of the target protein (Kalchman et al. 1996). In addition, the aggregation of Htt significantly increased by proteasome inhibitors treatment, such as lactacystin, indicating that the proteasome plays an

important role in Htt degradation. The mutated Htt protein (mHtt) was ubiquitinated but could not be transported to the protease in time, resulting in UPS overload and dysfunction, which caused protein aggregation into intranuclear inclusions. Htt interacts with two major chaperone families, HSP70 and HSP40, like other proteins with polyQ sequences, affecting the refolding function of misfolded proteins in intracellular quality control systems. At the same time, many chaperones were also recruited in the Htt aggregates, which also affected the UPS pathway and produced more misfolded proteins. Conversely, increasing the expression of HSP70 and HSP40 can alleviate this damage. Davies et al. also found many other proteins, such as ubiquitin, in the cytoplasmic aggregates and inclusions of HD transgenic mice, suggesting that Htt leads to cytoplasmic aggregation of proteins, thereby interfering with the distribution and physiological functions of these proteins (Davies et al. 1997). For example, ubiquilin 1, ubiquilin 2 and Tollip are found in mHtt aggregates of HD animal models and cell models. These proteins all contain a motif that binds to ubiquitin. When it forms aggregates with mHtt, it can affect the function of UPS in protein degradation progress, and interfere the normal function of neurons. It has also been found in HD transgenic animals that the activity of intracellular proteasomes is significantly decreased with age, accompanied by the formation of Htt aggregates in the N-terminal. If the abnormal protein and aggregates is not properly degraded, it may trigger apoptosis. This indicates that the aggregates are intrinsically cytotoxic and cause cell deficiency.

9.4.2 The Correlation Between Autophagy/Lysosomal Pathway and Huntingtin Protein

More than 90% of long-period proteins and disabled organelles are degraded by the lysosomal pathway. The protein aggregation mentioned above may be a protective mechanism for cells to resist the exposure of hydrophobic residues, but at the same time the aggregated proteins are more difficult to be degraded by the proteasome, so the autophagy/lysosomal pathway may become a compensatory mechanism of these denatured proteins. The study found that the activity of the autophagy/lysosomal pathway was altered in experimental animal models of AD, HD, and PD. Autophagic lysosomal activity was enhanced in the brain of HD patients, and the bilayer membrane structure of vesicle was also significantly increased. Similarly, in the case of oxidative stress, autophagic activity was significantly enhanced in striatum of HD transgenic mice. Rubinsztein et al. found that the mTOR was recruited in polyQ aggregates of mHtt in the cultured cells, transgenic mice and HD patients, thereby causing dysfunction of mTOR signaling pathway, similar results also exist in other diseases caused by PolyQ expansion (Rubinsztein et al. 2007). Since the activity of mTOR is inhibited, the autophagy/lysosomal pathway activity is thus enhanced. In 2002, Ravikumar reported that autophagy is involved in the degradation of truncated Htt fragments. In the process of Htt degradation, soluble Htt protein level is increased

when the autophagosome formation inhibitor 3-MA (3-methyl adenine) or dimethyl adenosine or autophagosome-lysosome fusion inhibitor bafilamycin A1 treatment (Ravikumar et al. 2008). The autophagy activator rapamycin increases the clearance of aggregates and reduces the soluble form of the mutant protein. Qin Zhenghong et al. found that inhibition of autophagy reduced cell viability, increased mHtt aggregation in cells, and activation of autophagosomes reduced Htt accumulation and Htt aggregate synthesis. These studies suggest that autophagosomes may be another pathway mediated the clearance of mHtt aggregates, in case of blocked proteasome degradation pathway. The autophagy is also involved in the formation of Htt bodies, which are found in lymphoma cells of HD patients and are absent in healthy person. In the HD cell model, Kegel first reported morphological evidence of activation of autophagosomes. Other researchers subsequently confirmed this finding. In addition, Qin Zhenghong et al. also showed that overexpression of Htt in vitro increased the cleaved activity of Cathepsins and Caspase-3, so that the splicing and degradation of Htt was further accelerated. And the expression of cathepsin D and cathepsin H were significantly increased in the autopsy of HD patients. These evidences indicate that the activity of the autophagy/lysosomal pathway is enhanced in HD. However, studies also found increased activity of caspase-3, mitochondrial damage and cell apoptosis when autophagic enhancement. Therefore, the autophagic function in HD progress is complex.

Protein post-translational modification plays an important role in targeting Htt to degrade via the autophagy/lysosomal pathway. Krainc et al. found that acetylation of the K444 residue of mHtt made mHtt more accessible to autophagosomes, with enhanced degradation of mHtt, thus ameliorated the neurotoxicity in striatum and cortex (Krainc 2010). Steffan et al. also reported that the clearance of modified Htt by proteasome and autophagy/lysosome was significantly enhanced after IKK phosphorylation. In addition, they also found that the phosphorylation of the 13th amino acid of Htt promotes Htt degradation in a manner similar to IKK-mediated degradation of IκBα and FOXO3a. Wild-type Htt enters the nucleus after phosphorylation, undergoes modifications such as acetylation, ubiquitination, and palmitization, and is then selectively degraded by proteasomes and lysosomes. The process of lysosomal degradation is regulated by proteins such as LAMP2a and Hsc70 and Atg7. These data suggest that chaperone-mediated autophagy (CMA) is also involved in the degradation of Htt. This selective autophagy process can greatly reduce the negative effects of cell damage caused by excessive activation of non-specific autophagy, thereby improving the degraded efficiency of mHtt. However, as level of LAMP2a in lysosomes gradually decreases with age, the activity of CMA will gradually decrease. Therefore, LAMP2a, which decreases with time, affects the degraded rate and extent of Htt. This leads to the adult onset of HD.

In addition, the researchers also found that mHtt activates autophagy while affecting the recognition of other substrates by the autophagy system, thus autophagic activity continues to increase, but blocked autophagic flow affects cellular homeostasis, leads to accumulation of intracellular aggregates and disabling organelles instead.

9.4.3 *Huntingtin Protein Regulation of Intracellular Autophagy*

The autophagy/lysosomal pathway is regulated by a variety of factors within the cell. Kegel et al. reported that expression of exogenous full-length Htt or its N-terminal fragment in mouse-derived striatal cells induced Htt-positive autophagic vesicles and enhanced autophagy/lysosomal pathway, with increased expression of Cathepsin D in the vesicles (Kegel et al. 2000). This suggested that mHtt expression accelerates the formation of vesicles, but impairs the clearance of vesicles. Previous studies found that Htt plays a major role in vesicles of transport of autophagy, knockdown Htt or Htt-associated protein 1 (HAP1) blocked the retrograde transport of vesicles. In addition, Htt mutation leads to dysfunction of autophagic vesicles transport and obscured normal fusion of autophagic vesicle and lysosome, thus caused mHtt aggregates, impaired autophagic flow and cytotoxicity.

Qin et al. found that Htt overexpression caused cytoplasmic transport of Beclin1 through interaction, thereby enhancing the cellular autophagy and degradation of mHtt (Wu et al. 2012). Yuan et al. found that the aggregates of mHtt highly associated with the expression of Beclin 1. The aggregates of mHtt caused decreased expression of Beclin 1, consistently, Beclin 1 knockdown inhibited the initiation of autophagic pathway thereby leading to mHtt aggregates. Importantly, clinical results showed that the expression of Beclin 1 is age-related and decreased expression of Beclin 1 during aging. The discovery of mHtt in autophagic regulation refueled the research in autophagy and neurodegenerative disease.

9.5 Conclusions

Regarding the use of autophagy as a target to explore the treatment of polyglutamine diseases, the main purpose is to use the autophagy pathway to clear the mutant proteins. The ubiquitin-proteasome system clears away these mutant proteins mainly before the accumulation of mutant proteins and cell damage. When the ubiquitin-proteasome system is overloaded or damaged, the autophagy pathway is the main pathway that mediated the clearance of cellular aggregates (Fig. 9.1). Autophagy is generally considered to be unable to clear the mutant proteins that accumulate in the neuronal nuclei, which may explain why nuclear aggregated proteins are more toxic to neuron. However, although Atg8/LC3 can only co-localize with a few aggregates of ataxin-1-Q85 present in the cytoplasm, increased aggregates of mutant ataxin-1 in autophagy-related proteins (ATG5 or ATG8) knockdown cells, indicating that autophagy can partly reduce the accumulation of nucleus. In addition, rapamycin, the agonist of autophagy, was also observed to induce autophagy and reduce the toxicity of other polyQ proteins in the SCA1 animal model, suggesting that the strategy of clearing mutations by regulating autophagy may be a therapeutic approach for SCA1.

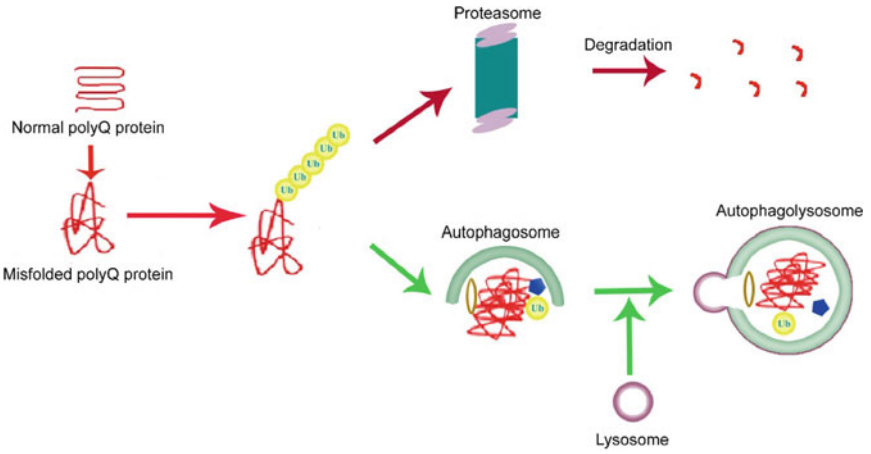

Fig. 9.1 The quality control of mutant polyQ protein

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Chapter 10
Autophagy and Epilepsy

Meihong Lv and Quanhong Ma

Abstract Epilepsy is a long-term neurological disease characterized by convulsions that can be recurrent. It is mainly caused by an imbalance between excitation and inhibition in the central nervous system. Currently, the pathogenesis is still unclear, although it may be related to changes in ion channels, neurotransmitters and glial cells. In recent years, increasing attention has been paid to the role of autophagy in the development of epilepsy. This chapter focuses on the role of the mTOR pathway in epileptogenesis and the relationship between autophagy, glycogen metabolism and Lafora disease and discusses the potential role of autophagy as a target for the treatment of epilepsy.

Keywords mTOR · Autophagy · Epilepsy

Epilepsy is a long-term neurological disorder characterized by recurrent convulsions. It is caused by the abnormal discharge of nerve cells in the cerebral cortex due to an imbalance between excitatory and inhibitory neurotransmission in the central nervous system. Epilepsy has a high incidence and mortality and causes serious damage to the patient's physical and mental health and quality of life.

Although epilepsy cannot be cured, 70% of seizures can be controlled by drugs. Traditional antiepileptic drugs, including phenytoin and phenobarbital, have certain clinical effects. However, there are many side effects, such as a high teratogenic rate and hyperactivity and inattention, which are difficult for patients to tolerate. New antiepileptic drugs (such as lamotrigine, levetiracetam, topiramate and oxcarbazepine) have the advantages of good efficacy and few side effects and are easily tolerated by patients (Stafstrom and Carmant 2015). For drug-refractory epilepsy, neurosurgery, nerve stimulation therapy or diet change can be used to relieve epileptic symptoms without drugs (Stafstrom and Carmant 2015).

The etiology of epilepsy is complicated, and the underlying mechanisms have not yet been fully elucidated. Various factors are associated with epilepsy. In younger patients, the most common causes are hereditary, congenital and developmental disorders. In older patients, brain tumors, stroke, cranial trauma, metabolic abnormalities

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and neurodegenerative diseases are the main influencing factors. The pathogenesis may include changes in ion channels, neurotransmitters and glial cells. Recent studies have emphasized the essential role of autophagy in epileptogenesis.

10.1 The Role of Autophagy in Epilepsy

Autophagy is a way for cells to self-regulate and maintain normal physiological functions. Cells can eliminate certain toxins, pathogens and misfolded proteins or damaged organelles through autophagy, thus protecting the cell from further damage. It also facilitates the recycling of substances and energy in cells. However, insufficient or excessive autophagy can cause damage to the cells. Gradually, autophagy has been understood as an important method of cellular quality control, and attention has been paid to its role in the process of epileptogenesis and development.

10.1.1 mTOR Pathway and Epilepsy

The abnormal activation of the mTOR pathway has been shown to cause a variety of epilepsy syndromes, including hereditary epilepsy and various acquired epilepsies. Tuberous sclerosis complex (TSC) is a common autosomal hereditary disease characterized by lesions of multiple organs, including the brain, skin, kidneys, eyes, and lungs (Curatolo et al. 2008). Up to 96% of cases of TSC are associated with epileptic symptoms (Jozwiak et al. 2000). TSC is caused by mutations of the tumor suppressor genes TSC1 and TSC2 (Curatolo et al. 2008). TSC1-deficient mice and TSC2-deficient mice exhibit severe epileptic symptoms, concomitant with the excessive activation of mTOR and impaired autophagy. Hyperexcitability has been detected in the brains of TSC patients and in neurons differentiated from induced pluripotent stem cells derived from TSC patients (Nadadhur et al. 2019; Wang et al. 2007). Reduced inhibitory synapse function has been identified to contribute to the hyperactivity of TSC1-deficient neurons (Bateup et al. 2013). However, it is worth noting that mTOR is actively involved in most key steps of neural development, such as the establishment of neuronal architecture, the maintenance of synaptic strength, and the generation of excitatory pyramidal neurons and inhibitory GABAergic neurons (see Chap. 11). The hyperexcitability observed in TSC patients may be a consequence of excessive mTOR activation in the developing brain. This idea is consistent with the clinical fact that the vast majority of TSC patients first develop epilepsy as infants (Chu-Shore et al. 2010). In this context, it is worth noting that a small fraction of patients do not show seizures until adolescence (Chu-Shore et al. 2010), which may be because the pathogenic effect of TSC inactivation is cell type-specific. The deletion of TSC1 in either neurons or astrocytes results in seizures (Meikle et al. 2008; Zeng et al. 2008). Although TSC1-deficient neurons exhibit reduced inhibitory synaptic

transmission (Bateup et al. 2013), the deletion of TSC1 in GABAergic interneurons does not result in spontaneous seizures (Fu et al. 2012; Wang et al. 2007).

Epilepsy caused by cortical malformation, such as focal cortical dysplasia type IIb (FCD type IIb), is a refractory epilepsy with a histopathology similar to that of TSC that is mainly characterized by neurons with abnormal morphology, specifically dysmorphic neurons (DNs) and balloon cells (BCs). Specifically, BCs in the brains of FCD patients exhibit an accumulation of lysosomes and autophagy-related proteins, including Beclin 1, LC3, ATG5, and ATG12, as well as the autophagy regulator DOR and the autophagy receptor P62, indicating that autophagy is impaired in FCD. This deficiency in autophagy can be reversed in vitro by inhibiting mTOR, suggesting that the aberrant activation of mTOR may directly lead to defects in autophagy in FCD type IIb (Yasin et al. 2013). In fact, mutations in either PTEN or DEPDC5 (which encodes GATOR1) have also been reported in patients with FCD type IIb (Schick et al. 2006; Tsai et al. 2017). Both PTEN and GATOR1 are suppressors of mTOR. PTEN is one of the tumor suppressor genes encoding plasma membrane lipid phosphatase, which antagonizes PI3K-Akt signaling upstream factors of mTOR. GATOR1 activates the GTPase RagA/B, thus inhibiting mTORC1 activity under static and low amino acid conditions (Bar-Peled et al. 2013). These lines of evidence suggest that excessive mTOR activation, which results in deficient autophagy, is associated with the FCD type IIb. Consistent with these lines of evidence, PTEN deficiency results in severe epilepsy in mice, concomitant with impaired autophagy (McMahon et al. 2012). These findings strongly suggest that mutations in mTOR or the molecules associated with its pathway may be involved in the pathogenesis of FCD type IIb and FCD type IIb-associated epilepsy.

In addition, in animal models of acquired epilepsy, such as models of kainic acid-induced epilepsy (Shacka et al. 2007) and a model of traumatic brain injury-induced epilepsy (Chen et al. 2007), the mTOR signaling pathway is also overactivated. Therefore, the abnormal hyperactivation of mTOR is generally considered to be one of the main causes of epileptogenesis. Consistent with this idea, clinical trials have shown that treatment with rapamycin (a powerful autophagy inducer) can reduce the frequency of seizures in patients with TSC (Franz et al. 2006). In contrast to traditional antiepileptic drugs, rapamycin has no direct effect on neuronal excitability (Ruegg et al. 2007) but exerts its antiepileptic effects by inhibiting the mTOR signaling pathway. Intervention with the mTOR inhibitor rapamycin not only reduces the number of epileptic seizures but also prevents or reverses the histopathological changes, thereby achieving antiepileptic effects (Wong 2010; Zeng et al. 2009).

Despite these findings, most data claiming that autophagy is involved in seizures are still indirect. As a highly conserved serine/threonine protein kinase, mTOR, in addition to suppressing autophagy, is also a critical regulator of transcription and translation. In mice with a specific deletion of PTEN in a subset of neurons, the levels of the potassium channel subunit Kv1.1, but not of Kv1.2, Kv1 or Kvβ2, in the hippocampus increased, and Kv1.1 distribution was also altered. These observations suggest that mTOR signaling may regulate neuronal excitability by modulating voltage-gated ion channel expression (Nguyen and Anderson 2018). Direct evidence that links autophagy and epilepsy comes from observations of ATG7 conditional

knockout mice. The deletion of ATG7 in mature neurons in the CaMKII-cre mouse line results in spontaneous recurrent seizures (McMahon et al. 2012).

10.1.2 Autophagy, the Metabolism of Glycogen and Lafora Disease

Lafora disease (LD) is an autosomal-derived lethal disease characterized by seizures, progressive myoclonus, cognitive impairment, and typical glycogen-like inclusion bodies, termed Lafora bodies (Ganesh et al. 2006). LD is the most common form of progressive myoclonic epilepsy. Patients generally die between the ages of 20 and 30. The disease is caused by mutations in the epilepsy-associated proteins laforin (encoded by the PME2A gene) or malin (encoded by the PME2B gene). Laforin is a glycogen 6 phosphatase that degrades the glycogen chain. Malin is an ubiquitin E3 ligase. Mutations in laforin and malin cause the abnormal accumulation of basophilic inclusion bodies (Lafora bodies), which are formed by the abnormal accumulation of glycogen, in the cerebral cortex, substantia nigra, globus pallidus and dentate nucleus. Knocking out the laforin gene, the malin gene, or both gene in mice recapitulates most of the symptoms of LD. Normal laforin inhibits the mTOR complex, allowing the autophagy machinery to function properly and maintain its function. In contrast, when laforin is mutated, the autophagy pathway is strongly inhibited by excessive mTOR activation, causing a dysfunction in glycogen clearance. Mutations in malin leads to failures in autophagosome formation. Unlike laforin-regulated autophagy, malin-regulated autophagy is mTOR-independent (Criado et al. 2012). Therefore, mutations in the laforin gene and the malin gene cause impaired autophagy and the accumulation of Lafora bodies in neurons. However, the mechanism by which Lafora bodies result in seizures is unclear.

10.2 Autophagy as a Target for the Treatment of Epilepsy

In summary, the overactivation of mTOR and insufficient autophagy may promote the development of epilepsy. Therefore, drugs targeting autophagy or mTOR signaling are expected to play a therapeutic role in epilepsy. As an inhibitor of mTOR, rapamycin plays an important role in the treatment of epilepsy. Firstly, the inhibition of the mTOR pathway by rapamycin can prevent seizures and improve the pathology of PTEN or TSC mutated mouse models caused by mTOR signaling disruption. For instance, data from the use of rapamycin in an animal model of TSC suggest that it can delay or inhibit the process of epilepsy by inducing autophagy, reduce the frequency of spontaneous seizures, and improve survival rate in mice (Zeng et al. 2011). Secondly, the addition of rapamycin to cortical sections of patients with cortical dysplasia reduced rhythm oscillations induced by the proconvulsant agent

4-aminopyridine, which blocks type A K^+ currents and enhances neurotransmitter release (Cepeda et al. 2010). Furthermore, high-dose rapamycin administration reduces cortical hyperactivation of the mTOR pathway, inhibits sputum, and partially improves cognitive deficits in infantile spasms epileptogenesis (Raffo et al. 2011). Finally, rapamycin may improve the development of epilepsy-related pathology and reduce the incidence of spontaneous epilepsy in a model of temporal lobe epilepsy (Zeng et al. 2009; Huang and Yang 2010). Although the effect of rapamycin depends on the time and duration of administration, and may depend on the model used, mTOR dysregulation is closely related to several hereditary and acquired epilepsy, and some of these epileptogenic processes can be reversed using mTOR inhibitors.

Some antiepileptic drugs, such as carbamazepine, also acts as an enhancer of autophagy, have also been reported to play a role in the metabolic disease alpha-antitrypsin deficiency. Treatment of carbamazepine reduces alpha-antitrypsin liver load and fibrosis. These results provide data for clinical trials of carbamazepine in patients with alpha 1-antitrypsin deficiency (Hidvegi et al. 2010). In addition, carbamazepine can improve cognitive impairment in Alzheimer's disease (AD) model mice by enhancing autophagic flow (Li et al. 2013). Alzheimer's disease patients have a higher frequency of seizures. The antiepileptic drug lamotrigine suppresses hyperexcitability in AD model mice (Zhang et al. 2014). Treatment with lamotrigine reduces the occurrence of epileptic spikes and attenuates cognitive deficits in AD model mice, possibly through the induction of autophagy via both mTOR-dependent and mTOR-independent manners (Wu et al. 2015; Zhang et al. 2014). In amyotrophic lateral sclerosis, the widely used antiepileptic drug valproic acid reduces key molecule TDP-25 induced neuronal toxicity by inhibiting endoplasmic reticulum stress-mediated apoptosis and enhancing autophagy (Wang et al. 2015).

One of the most striking features of autophagy is its ability to be induced under different conditions, such as nutritional deficiencies. Based on this feature, autophagy induced by nutritional restriction is considered a potential treatment for epilepsy. In this context, autophagy stimulation and caloric restriction under physiological conditions improves cognitive deficits in normal brain aging (Mattson 2010). Thus, many metabolic interventions for the treatment of epilepsy, including ketogenic diets, intermittent fasting, calorie restriction or specific diets, have been proposed (Hartman and Stafstrom 2013).

10.3 Conclusions

In this chapter, we discussed the relationship between autophagy and epileptogenesis and how autophagy-related pathways, especially impaired mTOR signaling pathways, lead to epilepsy. We also discussed the role of autophagy as a target of antiepileptic treatment. These studies suggest that the use of different drugs that activate autophagy, including potent mTOR inhibitors such as rapamycin, and mTOR-independent autophagy modulators, such as drugs or dietary ingredients, can be used

in the early treatment of epilepsy. They can prevent seizure recurrence and drug tolerance. Through the in-depth study of autophagy, the mTOR signaling pathway and the mechanism underlying epilepsy, new antiepileptic drug targets are expected to be found. Whether these targets act on the mTOR signaling pathway itself or on the other pathway of autophagy they will have a profound impact on the treatment strategy for epilepsy. Although autophagy is an important pathogenesis in epilepsy, the role of autophagy activation in epilepsy treatment remains to be explored.

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Chapter 11
Autophagy in Neurodevelopmental Disorders

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Abstract Neurodevelopmental diseases are a class of neurodevelopmental disorders characterized by cognitive impairment and behavioral abnormalities and are mainly manifested as developmental disorders of the brain and nervous system. The pathological mechanism is not fully understood and may be related to hereditary or environmental factors. The elevation of autophagy during neural development suggests that autophagy may be involved in the process of neurodevelopment. This chapter focuses on the important functions of autophagy in all aspects of neurodevelopment and the role and mechanism of autophagy in neurodevelopmental disorders, especially in autism spectrum disorder.

Keywords Autophagy · Neuronal development · Autism spectrum disorder · Neurodevelopmental disease

11.1 Autophagy Controls the Essential Steps of Neuronal Development

Neurodevelopmental diseases are a class of neurological disorders characterized by cognitive impairment and behavioral abnormalities and are often associated with abnormalities in the establishment and maintenance of brain circuits. Autophagy has been identified as an essential mechanism contributing to the pathogenesis of neurodevelopment since it actively controls/regulates the main steps of brain development. Therefore, this chapter briefly describes the roles of autophagy in brain development before discussing how autophagy is involved in the pathogenesis of neurodevelopmental diseases.

Autophagy is constitutively active in neurons even under nutrient-rich conditions (Boland et al. 2008; Lee et al. 2011). Neurons are postmitotic and highly polarized cells with axons, dendrites and synapses that form subcompartments with distinct molecular signatures. Autophagosomes are formed in distal axons and soma (Maday and Holzbaur 2016). Some studies have also observed autophagosome formation in

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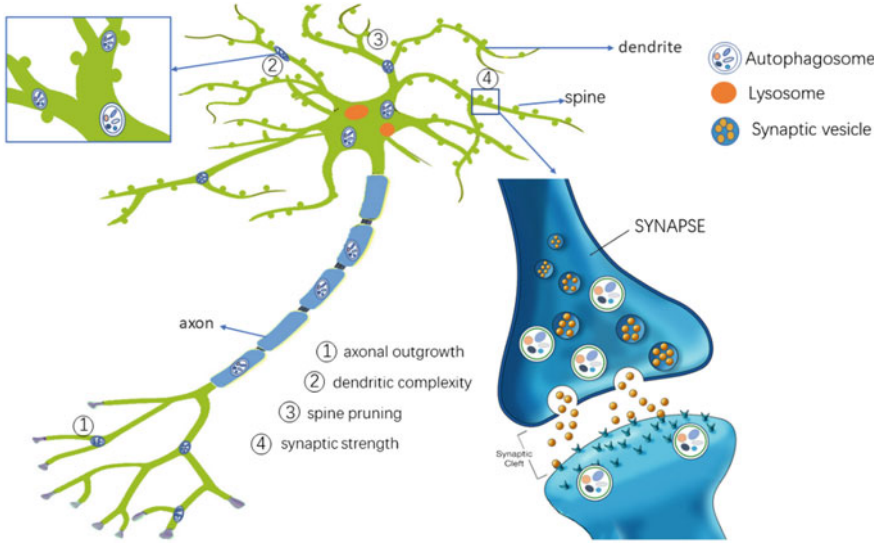

Fig. 11.1 Autophagy in neuronal development. Autophagosomes can be formed in the soma, axons, and dendrites, whereas mature lysosomes mainly reside in the neuronal soma. Thus, the autophagosomes formed in axons are retrogradely transported to the soma, where they fuse with lysosomes and degrade cargo. Autophagy is involved in the key steps of neuronal development that regulate neuronal size, axonal outgrowth, dendritic complexity, and spine pruning. Autophagy is also involved in the formation and maintenance of synapses

dendrites, especially in response to particular stimuli (Hernandez et al. 2012; Shehata et al. 2012). Autophagosomes form at axon terminals and rapidly move towards the neuronal soma without requiring an external stimulus such as starvation (Maday and Holzbaur 2014). Autophagosomes degrade cargo after arriving in the soma, where mature lysosomes reside (Maday et al. 2012). Neuronal autophagy is essential for the formation of appropriate neuronal connections and the maintenance of their functions thereafter (Fig. 11.1).

11.1.1 Autophagy Is Required for Neuronal Development

Neuronal autophagy is critical for the growth and refinement of the polarized architectures of neurons. The suppression of basal autophagy in developing neurons by the conditional deletion of ATG5 or ATG7, two proteins required for autophagosome formation, in a nestin-cre mouse line results in the accumulation of cytoplasmic inclusion bodies and protein aggregates in neurons, leading to neurodegeneration (Hara et al. 2006). The deletion of ATG7 in mature pyramidal neurons also causes neurodegeneration and is accompanied by hyperphosphorylated tau (Inoue et al. 2012). The neurodegeneration of these mature pyramidal neurons can be rescued by

suppressing the phosphorylation or deletion of tau (Inoue et al. 2012). However, the deletion of ATG7 in pro-opiomelanocortin (POMC) neurons results in the perturbation of axonal growth and thus reduced axonal projections (Coupe et al. 2012). The inhibition of Ulk1, which is required for autophagy induction, suppresses neurite outgrowth (Tomoda et al. 1999). Mammalian target of rapamycin (mTOR) serves as an inhibitor of autophagy. Suppressors of mTOR, such as TSC1/2 and PTEN, are essential regulators of neuronal size, axonal growth and dendritic development (Takei and Nawa 2014). However, another study observed that the knockdown of ATG7 by siRNA causes the elongation of axons via the RhoA-ROCK pathway (Ban et al. 2013). Dendritic spines are the primary location of excitatory synapses. Dendritic spines form during the early postnatal period, and while many spines are likely to be formed and removed throughout life, the net number is often gradually pruned during adolescence, with a stable level being reached in adulthood. Spine pruning is crucial for the establishment and function of mature neuronal networks (Riccomagno and Kolodkin 2015). Impaired spine pruning contributes to the pathogenesis of many neurodevelopmental disorders, such as autism spectrum disorder (ASD), schizophrenia, and fragile X syndrome (FXS) (Hutsler and Zhang 2010). Autophagy has been identified as a fine-tuning mechanism in neuronal development through its regulation of spine pruning. The loss of autophagy in neurons results in impaired spine pruning, which leads to aberrant neural circuits and eventually behavioral abnormalities (Tang et al. 2014a, b). In this context, microglial autophagy also plays essential roles in spine pruning. The deletion of ATG7 in microglia in a Lyz2-cre mouse line results in increased spine density through impaired spine pruning (Kim et al. 2017).

11.1.2 Autophagy Maintains Synaptic Plasticity

In addition to maintaining the structural development of neurons, autophagy also regulates the strength of synapses. The suppression of mTOR by rapamycin decreases the number of synaptic vesicles and the release of dopamine in an autophagy-dependent manner (Hernandez et al. 2012). Deficiencies in autophagy in dopaminergic neurons by the conditional ablation of ATG7 causes an increase in the size of axonal profiles, an increase in evoked dopamine release and rapid presynaptic recovery (Hernandez et al. 2012). Autophagy may be involved in degrading synaptic vesicles. Active Rab26, which localizes to the surface of synaptic vesicles and promotes the formation of these vesicles, can recruit Atg16L1, an autophagic protein essential for the formation of autophagosomes (Binotti et al. 2015). Brain-derived neurotropic factor (BDNF), a key player in synaptic plasticity, is crucial for maintaining baseline autophagic activity in the brain by suppressing autophagy. Moreover, the suppression of autophagy is required for BDNF-induced synaptic plasticity (Nikoletopoulou et al. 2017). The presynaptic protein Bassoon, which is an essential regulator of the release of synaptic vesicles, inhibits autophagy in presynaptic boutons via binding to ATG5 (Okerlund et al. 2017).

It is worth noting that synaptic autophagy has targets other than synaptic proteins and vesicles (Binotti et al. 2015; Nikoletopoulou et al. 2017), such as mitochondria, which play essential roles in the formation of synapses and the maintenance of synaptic function (Courchet et al. 2013; Kimura and Murakami 2014). Dysfunctional mitochondria are observed in many neurodevelopmental disorders, including ASD (Tang et al. 2013). Damaged or dysfunctional mitochondria can be cleared by autophagy, a process called mitophagy (Ebrahimi-Fakhari et al. 2016). In this context, mitophagy is required for spine pruning (Tang et al. 2014a, b).

11.2 Autophagy in Developmental Disorders

11.2.1 Introduction to ASD

ASD encompasses a highly heterogeneous set of neurodevelopmental conditions that are semantically and clinically grouped because they share common behavioral hallmarks, including abnormal social interactions, impaired communication skills and repetitive or stereotypical behaviors. ASD patients also have other associated symptoms, such as abnormal intellectual ability, irritability, anxiety, aggression, compulsions, mood lability, gastrointestinal issues, and depression and sleep disorders (Elsabbagh et al. 2012). ASD patients display wide variations in symptoms, severity and functional disability. ASD is caused by alterations in brain development. Due to a lack of biomarkers, the diagnosis of ASD is mainly based on behaviors. Clinical diagnosis is based on the presence of core symptoms, including impaired social interactions and repetitive or stereotypical behaviors. The Diagnostic and Statistical Manual of Mental Disorders is conventionally used as a gold standard for autism diagnosis (Elsabbagh et al. 2012).

Both genetic and environmental factors contribute to ASD etiology. Direct evidence of the strong genetic contribution to the risk of ASD has come from twin studies. Monozygotic twins have higher concordance rates for ASD (ranging from 60 to 90%) than dizygotic twins (from 0 to 30%) (Constantino et al. 2010; Hallmayer et al. 2011; Risch et al. 2014; Sandin et al. 2014). In families with a history of ASD, the likelihood of having a child with ASD increases with the proportion of the genome that the child shares with the affected sibling or parent (Constantino et al. 2010; Hallmayer et al. 2011; Risch et al. 2014; Sandin et al. 2014). Hundreds of genes have been identified as genetic risk factors for ASD. Despite their diversity, these genes, when mutated or deleted, contribute to ASD etiology via common pathways such as dysregulated mTOR signaling, an imbalance in excitatory/inhibitory neurotransmission, and impaired brain connections and synaptic plasticity (Geschwind 2008). The commonalities in the cellular and molecular mechanisms offer us opportunities to intervene in ASD.

Environmental factors such as inflammation during pregnancy, environmental toxicants, psychological stress, and nutrition have significant links to ASD as well. These

environmental factors can interact with susceptibility genes for ASD, and through epigenetic changes, may lead to alterations in gene expression, transgenerational epigenetic inheritance, or both (Liu et al. 2016; Lyall et al. 2014).

In this chapter, we will review the evidence that dysregulated autophagy caused by either genetic factors or environmental factors is linked to ASD.

11.2.1.1 Evidence that Dysregulated Autophagy Is Linked to ASD

ASD-associated exonic copy-number variants have been reported in the genes involved in autophagic pathways (Poultney et al. 2013). Impaired autophagy, which is evidenced by decreased levels of LC3-II, a marker of autophagosome formation, and elevated levels of p62, an autophagic substrate, has been observed in the brains of ASD patients (Tang et al. 2014a, b). Mutations in genes encoding autophagic proteins have been observed in ASD patients. mTOR, for example, one of the central suppressors of autophagy, is closely linked to ASD. Elevated mTOR activity, concomitant with impaired autophagy, has been observed in the brains of ASD patients (Tang et al. 2014a, b). Mutations in genes that inhibit mTOR activity, such as tuberous sclerosis complex 1/2 (TSC1/TSC2), NF1 (neurofibromatosis type 1), and PTEN (phosphatase and tensin homolog deleted on chromosome ten), lead to syndromic ASD with tuberous sclerosis, neurofibromatosis, or macrocephaly (Bourgeron 2009). Rapamycin, an mTOR inhibitor, induces autophagy and restores autistic behaviors in TSC2-deficient mice and PTEN-deficient mice (Tang et al. 2014a, b; Zhou et al. 2009). However, it fails to do so when autophagy is deficient (Tang et al. 2014a, b), indicating that hyperactive mTOR activity leads to ASD via impairing autophagy. Studies on autophagy-deficient mice further highlight a direct link between autophagy and ASD. Mice lacking ATG7, a key player in the formation of autophagosome, in both neurons and microglia exhibit autistic behaviors (Kim et al. 2017; Tang et al. 2014a, b). The absence of Wdfy3, which is required for the clearance of protein aggregates by autophagy, results in migratory defects of cortical projection neurons and an increase in the size of the cerebral cortex, which is pathologically characteristic of ASD (Orosco et al. 2014). Heterozygous deficiencies in Ambra1, an upstream factor of Beclin 1 and a key player in the formation of autophagosomes, results in autism-like behaviors in female but not male mice (Dere et al. 2014).

11.2.1.2 mTOR Signaling, Which Suppresses Autophagy, Plays Essential Roles in the Pathogenesis of ASD

mTOR is a protein serine/threonine kinase that belongs to the phosphatidylinositol 3-kinase (PI3K)-related kinase family. mTOR serves as an autophagy suppressor. mTOR-mediated autophagy is an essential mechanism underlying ASD etiology. Hyperactive mTOR is detected in the brains of ASD patients and is accompanied by impaired autophagy (Tang et al. 2014a, b). Mutations in genes that inhibit mTOR kinase, including TSC1 (aka hamartin), TSC2 (aka tuberin), neurofibromatosis type

1 (NF1) and PTEN, have been detected in some ASD patients(Bourgeron 2009). These genes suppress mTOR in distinct ways. TSC1/2 functions as a GTPase activating protein (GAP), accelerating GTP hydrolysis and thus inactivating Rheb in its GDP-bound form. GTP-bound Rheb (active Rheb), but not GDP-bound Rheb, can directly bind to mTORC1 and activate its kinase activity (Long et al. 2005). PTEN acts as a dual specificity phosphatase to curb mTOR signaling and inhibit both the PI3K/Akt and MAPK pathways (Zhou and Parada 2012). NF1 regulates mTOR activity by suppressing Ras, which is an upstream regulator of PI3K and ERK signaling. The latter, when activated, suppresses the TSC1/2 complex. Deficiencies in any of these genes in mice recapitulates autistic behaviors, including alterations in social interaction, learning and memory, and epilepsy (Kwon et al. 2006; Tang et al. 2014a, b), some of which can be reversed by rapamycin treatment (Kwon et al. 2003; Tang et al. 2014a, b; Zhou et al. 2009) in an autophagy-dependent way (Tang et al. 2014a, b). These findings support the hypothesis that the suppression of autophagy caused by hyperactive mTOR contributes to the pathogenesis of ASD.

11.2.2 Tuberous Sclerosis Complex (TSC)

Heterozygous mutations that abrogate the activity of the TSC1/2 protein complex cause TSC, which is clinically manifested by the appearance of nonmalignant tumors in multiple organs (Han and Sahin 2011). The neurological manifestations of TSC are autistic syndromes, including epilepsy, intellectual disability and social deficits. Clinical reports have shown that ASD is observed in 20–60% of TSC patients (Bolton et al. 2002; Wiznitzer 2004). TSC-associated ASD accounts for 1–4% of total cases of ASD. ASD is more commonly observed in TSC patients with cognitive impairment, although approximately 20% of TSC-associated ASD individuals still have normal intellectual ability (de Vries et al. 2007; Prather and de Vries 2004; Wiznitzer 2004).

TSC1-deficient mice and TSC2-deficient mice exhibit autistic behaviors including deficits in cognition and social interaction (Ehninger et al. 2008; Goorden et al. 2007), which are accompanied by abnormalities in neuronal architecture and differentiation, such as an increase in the size of neuronal soma, the aberrant production of multiple axons and an increase in dendritic spine density (Kwon et al. 2006; Tang et al. 2014a, b; Tavazoie et al. 2005). TSC1/2-deficient neurons exhibit impaired spine pruning, which can be reversed by rapamycin treatment, indicating a contribution of hyperactive mTOR. However, rapamycin fails to rescue impaired spine pruning in TSC1/2+/− neurons when autophagy is deficient, confirming that TSC1/2 regulates dendritic spine density through mTOR-dependent autophagy (Tang et al. 2014a, b). A recent study further observed that TSC1/2 regulates mitochondrial dynamics and metabolism, which are essential for the formation and strength of synapses. Tsc1/2-deficient neurons exhibit the accumulation of mitochondria in their cell bodies, but axonal mitochondria are depleted, which is concomitant with

impaired axonal and global mitophagy. Importantly, blocking mTORC1 or inducing mTOR-independent autophagy restores mitochondrial homeostasis (Ebrahimi-Fakhari et al. 2016). Another piece of evidence that supports the regulation of synaptic plasticity by TSC1/2 comes from electrophysiological analysis in TSC1/2-deficient mice. TSC2+/− mice display late phase long-term potentiation (LTP) following a single train of HFS that normally only induces early phase LTP (E-LTP), a transient form of LTP (Ehninger et al. 2008). TSC1+/− mice exhibit deficiencies in hippocampal mGluR-long-term depression (LTD) (Bateup et al. 2011; Hou and Klann 2004).

Consistent with the fact that ASD patients often also have epilepsy, hyperexcitability is observed in the brains of both TSC patients and the TSC1 conditional deficient synapsin-cre mouse line (Wang et al. 2007). Another study showed that the loss of Tsc1 results in hippocampal network hyperexcitability manifested by elevated spontaneous activity in dissociated cultures and increased seizure susceptibility in vivo. Hyperactivity in TSC1-deficient neurons is not due to changes in intrinsic and synaptic excitability but is due to reduced inhibitory synapse function, leading to an increased E/I balance (Bateup et al. 2013), an essential mechanism underlying ASD (Bourgeron 2009).

Hyperactive mTOR also leads to abnormalities in earlier cortical development by affecting embryonic neural precursor cells (NPCs) (Magri and Galli 2013). Mice in which TSC1 is deleted in all NPCs exhibit perinatal death, megalencephaly and reduced pup-mother interactions, which can be partially rescued by prenatal treatment with low doses of rapamycin (Anderl et al. 2011). Mice in which TSC2 is deleted in radial glial cells, from which pyramidal neurons are derived, exhibit postnatal megalencephaly, an increase in the generation of intermediate progenitor cells at the expense of early-born deep-layer cortical neurons, and die between 3 and 4 weeks of age (Way et al. 2009). The deletion of TSC1 in interneuron progenitors leads to the decreased generation of GABAergic cells of an enlarged size, which is accompanied by deficient autophagy (Fu et al. 2012).

Like TSC1/2, PTEN, a suppressor of mTOR, exhibits similar functions in brain development. PTEN-deficient mice exhibit abnormal social interaction, impaired cognition, seizures, macrocephaly and other morphological changes such as increased spine density and altered neuronal soma size (Kwon et al. 2003, 2006; Sunnen et al. 2011). PTEN also regulates synaptic function. PTEN-deficient mice exhibit deficits in both LTP and LTD (Wang et al. 2006). It is worth noting that the ablation of PTEN in hippocampal DG and CA3 neurons results in dysregulated LTP and mGluR-dependent LTD, which occurs before the onset of visible morphological abnormalities, indicating that PTEN regulates both neuronal architecture and synaptic function (Takeuchi et al. 2013).

11.2.3 Fragile X Syndrome (FXS)

FXS is the most common heritable form of intellectual disability and is a leading genetic cause of ASD. FXS is caused by the expansion of a poly-CGG trinucleotide repeat of ~50 to >200 repeats located in the 5′ UTR of the fragile X mental retardation 1 (Fmr1) gene, which results in the hypermethylation and epigenetic silencing of the Fmr1 gene (Yan et al. 2018). Fragile X mental retardation protein (FMRP), the gene product of Fmr1, is an RNA-binding protein. FMRP suppresses the translation of various mRNAs at synapses and is required for activity-dependent synapse elimination (Pfeiffer et al. 2010; Sharma et al. 2010). FMRP deficiency also results in hyperactive mTOR (Sharma et al. 2010). A recent study observed that the activation of autophagy by knocking down raptor, which inhibits mTOR activity, rescues deficits in synaptic plasticity and cognition in FMRP-deficient mice (Yan et al. 2018).

11.2.4 Autophagy May Be Involved in Environmental Factors Associated with ASD

Although further evidence is necessary, some studies have suggested a link between autophagy and environmental risk factors associated with ASD. Several environmental factors, such as advanced maternal age, maternal obesity, gestational diabetes, maternal autoimmunity, and maternal infections, are associated with an increased risk of ASD (Estes and McAllister 2015) through a common pathway, namely, maternal immune activation (MIA) (Ashwood et al. 2011; Patterson 2011). The induction of MIA in mice results in increased spine density, concomitant with altered levels of a microglial receptor (CX3CR1) (Fernandez de Cossio et al. 2017). In this context, microglial autophagy-mediated spine pruning is an essential mechanism contributing to ASD (Kim et al. 2017). Furthermore, the lack of branched chain amino acids (BCAAs) (Novarino et al. 2012) and the elevated expression of interleukin-17a (IL-17a) stimulated by MIA (Choi et al. 2016), both of which induce autophagy (Lynch and Adams 2014; Orosz et al. 2016; Zelante et al. 2012), results in autism-like behaviors in mice. These lines of evidence indicate that autophagy contributes to ASD pathogenesis.

Autophagy is also involved in the pathogenesis of other neurodevelopmental disorders, such as schizophrenia and epilepsy, which will be discussed in other chapters.

11.3 Conclusions

In this chapter, we described the roles of autophagy in neuronal development and the formation and maintenance of synapses. We discussed how autophagy is involved in

neurodevelopmental disorders, especially ASD. We demonstrated that autophagy-related pathways, such as the mTOR signaling pathway, are involved in the pathogenesis of ASD. All of these lines of evidence emphasize the pivotal role of autophagy in ASD. However, further investigations are needed to determine how mTOR-independent autophagy is associated with ASD, whether and how environmental risk factors cause autophagy-dependent ASD, and whether the activation of autophagy can act as a therapeutic strategy in neurodevelopmental disorders. Investigations into these questions will identify novel targets or strategies for the treatment of neurodevelopmental disorders.

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Chapter 12
Autophagy and Pituitary Adenoma

Zhebao Wu and Weiting Gu

Abstract Pituitary adenomas (PAs) are common, benign intracranial tumors that are usually effectively controlled with surgery, pharmacotherapy or radiotherapy. Some PAs against which conventional treatment is ineffective are great clinical challenges at present. Autophagy is a widespread physiological process in cells. Through autophagy, cells can degrade damaged or redundant proteins and organelles and achieve the recycling of intracellular substances to maintain the homeostasis of the intracellular environment. An increasing number of studies have demonstrated the importance of autophagy in tumor therapy. Both radiotherapy and chemotherapy can induce autophagy, which plays different roles in the course of therapy. In recent years, there has been growing interest in the role of autophagy during the treatment of PAs. This chapter reviews the recent progress of research on autophagy in PA and the autophagic mechanisms in the treatment of PA.

Keywords Autophagy · Pituitary adenoma · Dopamine agonists · Temozolomide · Autophagic cell death

12.1 Overview of Pituitary Adenomas

PAs are common benign endocrine tumors arising from the anterior pituitary, with a prevalence of approximately 7.5–15/100,000 and accounting for approximately 10–15% of intracranial tumors. Prolactinomas (40–60%) and nonfunctioning adenomas (20–30%) are the most common subtypes of PAs (Ezzat et al. 2004).

PAs are commonly divided into microadenomas (diameter $\leq$ 1 cm), large adenomas (diameter > 1 cm) and giant adenomas (diameter $\geq$ 4 cm). According to whether they secrete hormones, PAs can be divided into nonfunctioning adenomas and functional adenomas; the latter include growth hormone (GH) adenomas, prolactinomas, adrenocorticotropic hormone (ACTH) adenomas, thyroid-secreting hormone (TSH) adenomas, luteinizing hormone (LH) adenomas and follicle-stimulating hormone

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(FSH) adenomas. PAs are classified as invasive and noninvasive on the basis of imaging characteristics and biological behavior. The invasion of PA can be graded by the Knosp classification according to the relationship between PA and internal carotid arteries. According to the pathological characteristics of tumors, PAs can be categorized as benign and malignant (pituitary carcinoma and pituitary metastatic tumors); the latter is rare (Mete and Lopes 2017).

The treatments of PA include surgery, pharmacotherapy and radiotherapy. Dopamine agonists (DAs), including bromocriptine (BRC) and cabergoline (CAB), are the first choice for prolactinomas. Surgical resection, including transsphenoidal (endoscopic and microscopic) operations and craniotomy, is the primary choice for PAs except prolactinomas (Farrell et al. 2016). Messerer et al. found that the total resection rate of Knosp grade 2–3 tumors by microsurgery was 47.8 and 16.7%, respectively, while that by endoscopic surgery was 88.0 and 67.9% (Messerer et al. 2011). The remission rates of endoscopic transsphenoidal surgery for pituitary macroadenoma and invasive PAs were 69% and 40%, respectively. Obviously, conventional treatment is ineffective against a considerable fraction of PAs, which is a challenge in the clinic.

12.2 Autophagy and Normal Pituitary Gland

Autophagy is a process of forming autophagosomes by wrapping long-lived proteins and discarded organelles inside a cell with a double layer of membrane. Then, the autophagosomes fuse with lysosomes to form autophagolysosomes and degrade the contents and recycle the substances in the cells. It is a widespread physiological process that can remove damaged or redundant proteins and organelles from cells by degradation and realize the recycling of intracellular substances to maintain the homeostasis of the intracellular environment. Autophagy, involving more than 30 core autophagy-related genes (Atg), is a tightly regulated multistep process that is closely related to cell homeostasis, differentiation, proliferation and apoptosis (Klionsky and Emr 2000).

The discovery of autophagy in normal pituitary tissue was first reported in pituitary lactotrophs. In the secretory cells of the anterior pituitary, crinophagy was found to be a method of degrading excessive secretions (Weckman et al. 2014). In 1981, Poole et al. found that excess prolactin (PRL) granules would fuse with the lysosomes to be degraded and that the amino acids in the peptides would be recycled if the rats failed to conceive in the natural estrus cycle (Poole et al. 1981). Bernabe et al. found that PRL secretion increased during lactation and that excess PRL secretory granules were degraded by crinophagy after weaning (Bernabe et al. 2001). The mechanism of crinophagy in lactotrophs may be related to the levels of steroid hormones such as estradiol and progesterone (Farquhar 1971). Crinophagy was also found in other types of pituitary cells, such as corticotropic cells, gonadotropin cells, GH cells and thyrotropin cells (Farquhar 1969; Moi et al. 1984; Sirek et al. 1976). Moi et al.

found that crinophagy increased in the corticotropes during states of both hyposecretion induced by dexamethasone and hypersecretion due to adrenalectomy (Moi et al. 1984). In pituitary cells, crinophagy regulates hormone levels in physiological and pathological states (Smith and Farquhar 1966). Autophagic vacuoles containing rough endoplasmic reticulum and ribosomes were found in lactotrophs in the anterior pituitary, but few secretory granules were found (Smith and Farquhar 1966). The volume of autophagosomes increased with the decrease in rough endoplasmic reticulum and Golgi surface area (Poole et al. 1981). Autophagy was also more active in the externally induced lactotroph degeneration model (Smith and Farquhar 1966). Accordingly, the autophagy system was primarily responsible for the conversion of secretory protein synthesis mechanisms in the normal pituitary (Kuriakose et al. 1989).

12.3 Autophagy and Pituitary Tumors

Similar to the study of normal pituitary autophagy, there are few studies on autophagy and PA. Only a few cases have been reported. In 1977, Kovacs et al. found by electron microscopy that autophagy degraded secretory granules to form pigment granules, which are recurrent characteristics of rat spontaneous prolactinoma (Kovacs et al. 1977). Subsequently, Kovacs et al. reported another case of a silent ACTH adenoma patient whose tumor specimens showed crinophagy (Kovacs et al. 1978). Kovacs et al. thought that the intracellular degradation of secretory granules by autophagy or crinophagy was responsible for the normalization of ACTH in this patient.

In a study of 300 PA patients, Horvath et al. (1980) found that in 17 cases of silent ACTH adenomas, only 2 cases showed signs of increased autophagy. Autophagy was one of the reasons for the "silence" of PA patients. In 1982, Mashiter et al. (1982) reported a case of pituitary macroadenoma with evident acromegaly, but the serum GH level of the patient was low. After excluding the cause of pituitary apoplexy, Mashiter et al. believed that the increase in GH secretory granules degraded by GH cells through crinophagy was the cause of this contradiction. At present, the roles of autophagy in the occurrence and development of PAs are unclear, and single, nonrepeated case reports are not enough to reveal the mechanism of autophagy in PA. More research is needed in the future (Weckman et al. 2015).

12.4 Autophagy in Pharmacotherapy of Pituitary Adenomas

Autophagy has different effects in the course of tumor treatment. On the one hand, autophagy can induce tumor cell death and play a therapeutic role. On the other hand, tumor cells can degrade damaged proteins and organelles by autophagy, which

promotes cell survival and makes them insensitive to treatment. More and more studies have demonstrated the importance of autophagy in the treatment of tumors, and its therapeutic role in PA will gradually attract more attention.

12.4.1 Dopamine Agonists

Currently, DAs are the first choice for prolactinomas. Both BRC and CAB are effective for controlling clinical symptoms, reducing PRL level and shrinking the tumor volume (Colao and Savastano 2011; Huang et al. 2018; Wu et al. 2006, 2008). In a retrospective study of 455 prolactinoma patients treated with CAB, normal serum PRL level was achieved in 86% of the patients: 92% of the 244 patients with idiopathic hyperprolactinemia or microadenomas and 77% of the 181 patients with macroadenomas. Side effects occurred in 13% of the patients, but only 3.9% of the patients were discontinued from the CAB due to the side effects (Verhelst et al. 1999). In addition to prolactinomas, CAB is also used to treat GH adenomas, ACTH adenomas, and nonfunctioning adenomas (Feelders and Hofland 2013; Greenman et al. 2016; Katznelson et al. 2014; Wang et al. 2012).

Previous studies showed that DAs mainly inhibited the expression and transcription of the PRL gene by selectively activating dopamine receptor D2 (DRD2), thus reducing the synthesis and secretion of PRL (Mooney et al. 2016). DAs can also induce apoptosis by activating DRD2 to shrink the tumor volume (Beaulieu and Gainetdinov 2011; Colao et al. 2000). In addition, CAB can activate caspases via the ERK, JNK and p38 MAPK signaling pathways to induce apoptosis (Al-Azzawi et al. 2011; An et al. 2003; Radl et al. 2011).

DAs are capable of inducing autophagy in tumor cells. The autophagy marker LC3 was significantly expressed in BRC-treated prolactinomas. In vitro experiments confirmed that BRC increased the conversion rate of LC3-I to LC3-II, decreased the level of PRL, decreased the level of apoptosis and induced autophagic cell death (Geng et al. 2017). CAB also activated autophagy (Junn and Mouradian 2001). Recent studies have updated our understanding of DA mechanisms. CAB induced the formation of autophagosomes in pituitary tumor cells by inhibiting the mTOR signaling pathway. In addition, CAB inhibited autophagic flux by reducing the pH value of lysosomes. As a result, p62 protein and unfused autophagosomes accumulated in tumor cells, which eventually led to autophagy-dependent cell death (Geng et al. 2017; Lin et al. 2015). A further study by Leng et al. (2017) found that CAB induced autophagic cell death by activating dopamine receptor D5 (DRD5). The activation of DRD5 inhibited SOD1, increased intracellular ROS, induced autophagy by inhibiting the mTOR pathway, and blocked autophagy flux (Fig. 12.1).

Recently, Tang et al. found that BRC and CAB elicited cell death via different pathways (Tang et al. 2019). BRC induced the apoptosis of prolactinoma cells through the ERK/EGR1 signaling pathway, whereas CAB induced autophagic cell death by inhibiting AKT/mTOR signaling pathway. Zhang et al. observed that both CAB and the mTOR inhibitor everolimus inhibited GH3 cell proliferation and PRL secretion as

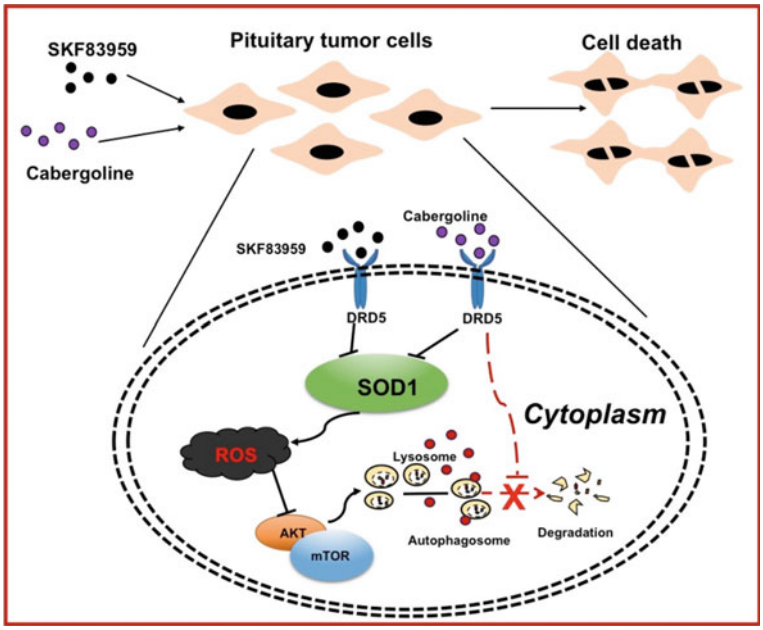

Fig. 12.1 CAB and the DRD5 agonist SKF83959 induce autophagic cell death by activating DRD5. The activation of DRD5 inhibits SOD1, increases intracellular ROS, induces autophagy by inhibiting the mTOR pathway, and blocks autophagy flux by decreasing the pH value of lysosomes

single agents, and a synergistic effect was noted with combination treatment only on inhibition of PRL secretion and not proliferation (Zhang et al. 2019). Wu et al. (2018) found that miRNA-93 targeting ATG7 regulated the sensitivity of prolactinomas to CAB treatment. Downregulation of miRNA-93 increased ATG7 protein expression and promoted MMQ and GH3 cell autophagy, resulting in autophagic cell death and eventually improving the effect of CAB.

12.4.2 Somatostatin Analogues

For GH adenomas and TSH adenomas, drug therapy is a supplementary treatment in patients who are unable to tolerate surgery, who have inoperable tumors, who have residual tumors or who have not achieved endocrine remission. The preferred drug is a somatostatin analogue (SRL), such as octreotide, lanreotide or pasireotide (Colao et al. 2016; Gadelha et al. 2014; Mooney et al. 2016; Yamada et al. 2014). Gadelha et al. (1987) exposed several pituitary GH adenomas from acromegaly patients to a SRL, SMS 201–995, for 10 days and found that autophagy occurred in tumor cells and GH secretion was inhibited. This finding suggested that SMS 201–995 regulates the intracellular degradation of GH secretory granules by mediating autophagy, thus

changing the serum GH level and achieving a therapeutic effect. Dagistanli et al. conducted a retrospective study on GH tumor specimens of 11 patients treated with SRL and 9 patients without SRL treatment (Dagistanli et al. 2018). The levels of TUNEL, caspase-3 and Atg5 were significantly increased after SRL treatment, while Beclin-1 and Ki-67 were significantly decreased. This finding suggested that SRLs could induce tumor cell apoptosis, increase autophagy and reduce cell proliferation.

12.4.3 Glucocorticoid Receptor Antagonist

Mifepristone (MF) is the only glucocorticoid receptor antagonist (GRA) currently available for clinical use. Although MF effectively controlled the symptoms of Cushing's disease, it required long-term imaging monitoring due to the risk of increasing tumor size (Dang and Trainer 2007; Feelders and Hofland 2013). In ovarian cancer cells, MF upregulated two key genes, GRP78 and CHOP, both involved in the unfolded protein response (UPR). In addition, MF was capable of promoting the accumulation of LC3-II and increasing the autophagy flux. When combined with chloroquine, MF induced ovarian cancer cell apoptosis (Zhang et al. 2016).

12.4.4 Temozolomide

Temozolomide (TMZ), a new oral alkylating agent, is the first-line choice for malignant glioma chemotherapy. LC3-II levels increased in TMZ-treated tumor tissues of rat glioma model (Aoki et al. 2008). TMZ induced autophagy in tumor cells and inhibited cell activity and migration (Kanzawa et al. 2004; Palumbo et al. 2012). In in vitro cell experiments, the effect of TMZ was improved by perturbing the autophagy process. 3-Methyladenine (3-MA) inhibited the anti-tumor activity of TMZ, while bafilomycin A1 (BafA1) enhanced the cytotoxicity of TMZ (Kanzawa et al. 2004). A recent study showed that 3-MA enhanced the cytotoxicity of TMZ combined with curcumin, whereas 3-MA prevented cell death caused by the combination of TMZ and cannabinoids (Torres et al. 2011). It was found that the combination of 3-MA and TMZ inhibited the death of tumor cells, but 3-MA alone promoted the death of TMZ-treated tumor cells (Hombach-Klonisch et al. 2018). 3-MA blocked the TMZ-induced ATP increase, which increased non-apoptotic cell death associated with micronucleus formation. These results suggest that the TMZ-induced autophagy-associated ATP surge is a cytoprotective mechanism leading to drug resistance (Katayama et al. 2007).

TMZ was first proposed for the treatment of pituitary cancer and invasive adenoma in 2006 (Lim et al. 2006). Currently, TMZ is regarded as the recommended treatment for invasive PAs that are refractory to conventional treatment (Almalki et al. 2017; Chatzellis et al. 2015; Liu et al. 2015a; Raverot et al. 2018). Although TMZ had shown excellent anti-tumor effects in some invasive PAs, it was effective in only 60% of the

published cases. The rest of the invasive PAs did not respond to TMZ treatment, and some of them even gained resistance after treatment (Syro et al. 2011). Kun et al. (2016) found that the activity of GH3 cells decreased by 25% after treatment with 100 mmol/L TMZ, while the activity decreased by 60% when combined with the autophagy inhibitor 3-MA. The ratio of LC3-II/LC3-I in GH3 cells increased, and the level of p62 decreased. HIF-1α knockdown inhibited TMZ-induced autophagy by means of blocking autophagy flux by neutralizing the lysosomal pH value in rat GH3 cells and thus increasing the antitumor efficacy of TMZ.

12.4.5 Combination Therapy of Autophagy Inhibitors

An increasing number of studies have shown that autophagy is an important factor leading to drug resistance in tumor treatment (Amaravadi et al. 2011). Autophagy plays different roles in different tumors and in the same tumor at different stages (Shintani and Klionsky 2004). Most tumors have been in the middle or late stage at the beginning of clinical treatment, when autophagy usually plays a protective role in the process of treatment and affects the clinical prognosis. The combination of chemotherapeutic drugs and autophagy inhibitors may enhance the sensitivity of tumors to chemotherapy. Fifty-one clinical trials related to "cancer and autophagy" that aimed to improve the sensitivity of chemotherapy and the prognosis of cancer patients by inhibiting autophagy have been registered at *Clinicaltrials.gov*. Chloroquine (CQ) and hydroxychloroquine (HCQ) are the only two drugs currently used in clinical treatment to inhibit autophagy. CQ is the most common autophagy inhibitor in clinical trials. Previous studies showed that CQ combined with chemotherapy prolonged the median survival and reduced mortality in patients with glioma (Rubinsztein et al. 2012). In the late stage of autophagy, the degradation of autophagic lysosomes is dependent on the activity of enzymes in lysosomes. These drugs mainly inhibit the H^+-ATPase activity of lysosomes to increase lysosomal pH to inhibit lysosomal enzyme activity and autophagosomal fusion with lysosomes (Sotelo et al. 2006). Lin et al. (2017) found that the combined use of CQ and CAB significantly increased the sensitivity of PA cells to CAB and was also effective in CAB-resistant PAs. CQ exacerbated CAB-mediated p62 and LC3-II accumulation and recruited caspase 8 to form a complex and finally induce cell death.

In clinical trials, the effects of combination therapy have varied widely. They have depended on whether the tumor cells were autophagy-dependent cells. Several mechanisms of autophagy dependence that may help to identify autophagy-dependent tumors have been demonstrated. Current studies suggest that RAS and BRAF mutations are associated with autophagy dependence (Mancias and Kimmelman 2011; Thorburn and Morgan 2015). RAS and BRAF are expected to be new markers of autophagy inhibition therapy in the future.

12.5 Autophagy and Radiotherapy for Pituitary Adenomas

Radiotherapy is mainly used for postoperative residual PA, recurrent PA and patients who are intolerant or refuse surgery. Autophagy plays an important role in radiotherapy. Several studies showed that radiotherapy induced autophagy in tumor cells via a PI3K/Akt/mTORC1 mechanism (Daido et al. 2005; Paglin and Yahalom 2006; Wang et al. 2014). Autophagy induced both radioresistance to protect tumor cells and type II programmed cell death to kill tumor cells. Lomonac et al. suggested that autophagy was an important cause of radioresistance in glioma stem cells and that inhibition of autophagy increased the sensitivity of glioma stem cells to radiotherapy (Lomonaco et al. 2009). Chaachouay et al. (2011) found that the aggregation of LC3-II was significantly reduced in the 3-MA-treated human breast cancer cell line MDA-231 after radiotherapy. Zhuang et al. found that rapamycin induced autophagy in glioma-initiating cells and increase their sensitivity to radiotherapy (Zhuang et al. 2011). Both autophagy inhibitors and autophagy catalysts were capable of increasing the sensitivity to radiotherapy in previous studies (Chiu et al. 2012, 2013; Han et al. 2014; Palumbo et al. 2012). Regulation of autophagy-related genes such as Atg7, Beclin-1 and TRAV6 enhanced the sensitivity of tumor cells to radiotherapy (Chiu et al. 2015; Liu et al. 2015a; Palumbo and Comincini 2013). The mechanisms of autophagy in radiotherapy of pituitary tumors remain to be further investigated (Xin et al. 2017).

12.6 Conclusions

In summary, PAs are mostly benign tumors that usually grow slowly; however, therapy of refractory or invasion cases is still challenging. The mechanisms of autophagy in the development and treatment of PA remain uncertain and need further elaboration. Obviously, autophagy will play a critical role in the individualized comprehensive treatment of PA in the future. It may be necessary to identify whether, when and in which direction we should try to manipulate autophagy during PA therapy. A better answer is needed to improve the outcomes of PA.

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Chapter 13
Autophagy and Schizophrenia

Yuexiong Yang and Lin Xu

Abstract Schizophrenia (SCZ) is characterized by abnormal thoughts, behaviors and speech, along with a decreased perception of reality that can included visual or auditory hallucinations, withdrawal of social activity and lack of motivation, etc. Many hypotheses related to the causes of SCZ have been proposed, but the underlying neuropathological mechanism remains unclear. Recent studies have suggested that there is an association between autophagy and SCZ. The strongest evidence for this comes from the expression of ATGs in the BA22 of postmortem samples from SCZ patients, coinciding with some of the brain imaging studies and certain hypotheses about SCZ in interpreting the positive symptoms. Autophagy dysfunction in the hippocampus, especially in the CA2 region, may relate to deficits of social communication and interaction in SCZ patients. mTOR regulation of autophagy is also potentially a piece of strong supporting evidence for the autophagic neuropathogenesis of SCZ. In vitro studies show that antipsychotics often induce autophagy through distinct mechanisms of drug action, but they may all share common features as autophagy inducers and antagonists of dopamine receptors.

Keywords Schizophrenia · Autophagy · Hippocampus · Brodmann area

13.1 Introduction

Psychiatric or mental disorders are serious health problems that affect thoughts, feelings, abilities, and behaviors. Schizophrenia (SCZ) is one of the common psychiatric disorders; it is a leading cause of disability and death and places an enormous burden on society worldwide. The neuropathology of SCZ remains unclear. The current treatments including medications, psychotherapies, and physiotherapies are far from satisfactory. Lacking a standard for biological diagnosis, its diagnosis still depends

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on mental scales and doctor experience, and there is a critical shortage in regard to research into its etiology and clinical treatments.

In this chapter, we will introduce how autophagy and its related mechanisms may link with at least part of the neuropathology of SCZ.

13.2 Schizophrenia

The English word schizophrenia (SCZ) was coined and introduced to the world by Dr. Eugen Beuler in 1908 to describe a serious health condition resulting in unusual behaviors. We know that SCZ is characterized by abnormal thinking, strange behaviors, and speech, and a decreased ability to understand reality, including hallucinations such as hearing voices or seeing things that do not exist, withdrawal from social activity, and lack of motivation, etc. Keeping this in mind, no SCZ patients show exactly all of these symptoms or exactly the same compositions of these symptoms. Thus, there is an agreement that SCZ is not a single disease and is instead a class of diseases. The interaction between environmental and genetic factors is perhaps the only generally accepted assumption for understanding the origin of SCZ and many other diseases.

The lifetime prevalence of SCZ is approximately 3.8–8.4%. In China, it is approximately 4.13%. Many SCZ patients have thoughts of or attempt to commit suicide, and 10–24% of suicide deaths are likely due to SCZ. Because SCZ is a chronic disease and one of the leading causes of disability and death, it places a heavy disease burden on society worldwide.

Many hypotheses regarding SCZ have been proposed, but the underlying neuropathological mechanism still remains unclear. Recent studies have suggested an association between autophagy and SCZ, which is a new research direction that may allow us to understand at least part of the neuropathology of SCZ.

13.3 The Hypotheses of SCZ

13.3.1 Nature Versus Nurture Hypothesis

It has been a long debate as to whether this disease is determined by nature or nurture. For infectious diseases, the pathology is clearly due to viruses, bacteria, and microorganisms, etc., but for SCZ it is more complex because no clear pathology has yet been identified. Although inheritability may be up to 80% in SCZ, identical twin studies have consistently revealed that there is only about a 40–50% concordance rate in identical twins (homozygous twins) in regard to SCZ. This concordance rate is largely reduced to 10–15% in heterozygous twins. Thus, genetic factors appear to be critical but still are only part of the etiology of SCZ. Genome-wide association

studies (GWAS) have found that tens and even hundreds of genes are significantly associated with the risk of suffering from SCZ, but different GWAS results seriously lack consistency, possibly due to the diverse genetic background of different populations, a lack of a biological diagnosis of SCZ or the heterogeneity of SCZ pathology.

Thus, the pathology of SCZ is suggested to be possibly due to an interaction between environmental and genetic factors (Misiak et al. 2017, 2018). However, environmental factors have also been difficult to identify. Clinical association studies have suggested that perinatal complications, abuse of addictive drugs, traumatic brain injury, difficulties during birth, poor nutrition during pregnancy, and early maternal separation, etc. may all increase the risk of developing SCZ. In contrast, later-onset cases of SCZ may be due to completely different reasons. There is evidence showing that infections of pregnant women, including toxoplasma infection, may cause SCZ. Epidemiologic studies have indicated that taking addictive drugs such as marijuana are also risk factors leading to injury of the prefrontal cortex and the temporal cortex that are known to play important roles in cognitive functioning (Olver et al. 2009). Deficits in cognitive functions are likely to be the core symptoms of SCZ. It is notable that different research fields have their own definitions of cognitive functions. Here, we believe that the cognitive deficits in thoughts, feelings, and abilities should have organized abnormal behaviors in SCZ.

13.3.2 Dopamine Hypothesis

Electroconvulsive therapy (ECT) and psychotherapies have long been used to treat SCZ and are still widely used. However, these treatments are of little help in understanding the neuropathology of SCZ. During the 1950s, its treatment was revolutionized with the development and introduction of chlorpromazine. Chlorpromazine and other typical antipsychotics are primarily blockers of dopamine D2 receptors. Overactivation of D2 receptors is thus suspected to be associated with the symptoms such as auditory or visual hallucinations (Lee et al. 2015). For example, D2 receptors in the auditory and visual association cortex (Brodmann Areas 20, 22, 37, 39 and 42) may mediate the hallucination and thus blockers of D2 receptors can be used to treat SCZ. However, this is an oversimplification of the mechanism of chlorpromazine action because chlorpromazine is also called "wintermin", able to cause hibernating sleep, so that it could just be temporarily stopping the symptoms of SCZ. Other neurotransmitter systems are also affected by chlorpromazine. Nevertheless, it is helpful for understanding and treating SCZ. In the 1990s, olanzapine with a similar mechanism of action was approved by the Food and Drug Administration (FDA) of the United States. It acts as the antagonist or inverse agonist on a number of receptors in the central nervous system including antagonism action against all of the dopamine receptors. So far, olanzapine is regarded by clinical doctors as one of the best medications, but it causes many side effects such as metabolism-associated disorders. From the perspective of neuroscience, chlorpromazine, olanzapine, and

other antipsychotics may just stop the working of the brain, e.g., the thinking and hallucination of SCZ patients, so that their behaviors stop being a danger to society anymore. This kind of treatment is far from satisfactory.

More recent studies have found that dopamine D1 receptors are also involved in SCZ, especially in the hippocampus and the prefrontal cortex that are known to play critical roles in learning and memory as well as in attention, working memory, thinking, executive function, etc., some of which are severely impaired in SCZ, and lead to disability in daily life and work. Thus, D1 receptors should be activated rather than inhibited. In agreement with this assumption, brain imaging studies have also found that the volume of the hippocampus or the functional connection of the prefrontal cortex including Brodmann area 10 (BA10) is significantly reduced in SCZ compared with healthy controls.

It is not clear yet but the unbalance of dopamine D1 versus D2 activation seems to be at least part of the key mechanism responsible for certain symptoms of SCZ. However, we need to address what causes the unbalance of dopamine D1 versus D2 activation. We may regard autophagy and its related mechanisms as a refuse disposal plant in human society, dealing with garbage in neurons and providing renewable energy to keep neurons in a healthy condition. This could be one of the best explanations as to why dopamine D1 versus D2 activation is unbalanced in SCZ.

13.3.3 Other Hypotheses

In this chapter, we are not going to introduce more hypotheses about SCZ in detail. However, a fact that is worth mentioning is that there is not yet a generally accepted hypothesis. From different angles or levels of the brain, the hypotheses of SCZ including neurodevelopment, synaptic plasticity, synaptic function, and neurotransmitter or neuromodulator systems, early life stress, etc. have been developed, for each of which a wealth of evidence has also been provided. Bearing this in mind, the bottleneck of this research field is the standard for biological diagnosis that requires a better understanding of the neuropathology of SCZ. Without such a fundamental basis, we do not even know whether we are exactly studying and discussing the same disease. Furthermore, SCZ is currently diagnosed as a class of diseases with diverse impairments of cognitive functions all still sharing certain common features. Furthermore, these common features are also partly shared by other psychiatric and neurological disorders. Thus, we think that the underlying mechanisms may not be specific in regard to physiological and pathological conditions. Due to this reason, autophagy and its related mechanisms may be one of the best candidates.

Consistent with this idea, one of the most famous hypotheses is the neurodevelopmental hypothesis of SCZ that was proposed in the 1990s (Zec and Weinberger 1986; Lewis et al. 1987). This hypothesis provides a valuable framework in that this disorder is at least partly due to a consequence of events occurring during early brain development. Furthermore, this hypothesis is also useful for understanding other mental disorders such as autism spectrum disorders (ASD), intellectual disability,

attention-deficit hyperactivity disorder (ADHD), and even Alzheimer's disease (AD). Another hypothesis consistent with the same idea is the synaptic hypothesis of SCZ because the brain regions responsible for SCZ have not been fully identified yet, although some brain regions such as the Brodmann 22, 10, and the hippocampus, are known to be critical for cognitive functions and are suggested to be associated with SCZ. Thus, Frankle WG proposed the following hypothesis in 2003: the fundamental pathology of SCZ may engage a series of factors leading to synaptic dysfunction, which further causes abnormal connectivity especially among the neural circuits including the prefrontal cortex, the limbic system, the striatum, and the thalamus, etc. (Frankle et al. 2003). Since synaptic functions involve neurotransmitters and their receptors such as glutamate and its receptors, 5-HT and its receptors, GABA and its receptors, and glial cells, which may all be critical for modulating synaptic transmissions, many related hypotheses have also been proposed. These hypotheses may relate to the changes that occur as a consequence of environmental factors (Lau et al. 2013).

It seems clear that the neuropathology of SCZ is not understood yet. Based on the scales of SCZ diagnosis, SCZ is likely to be a class of heterogeneous disorders, and these hypotheses are all likely reasonable.

13.4 Autophagy in SCZ

Autophagy is a quality control process of intracellular functions mediated by degrading intracellular components in lysosomes. Neurons, similar to any other cells, require a delicate balance between synthesis and degradation of proteins to ensure that misfolded proteins and damaged organelles can be rapidly identified and eliminated. Failure of this quality control results in the accumulation of dysfunctional proteins and organelles, leading to toxicity to neurons, precipitating the loss of neuronal functions, and even causing neuronal death (Morimoto and Cuervo 2009). Recent investigation has revealed a novel role of autophagy in mental disorders, especially autophagy malfunction in SCZ.

Autophagy involves a complex process such as sensing, sequestering, and targeting of the cargo, i.e., misfolded proteins and damaged organelles, etc., followed by delivery of the cargo to lysosomes through macroautophagy, chaperone-mediated autophagy, and microautophagy (Yang and Klionsky 2010). More than 30 autophagy-related genes (ATGs) have been identified that are involved in these steps of autophagy. The core autophagic machinery includes signal transduction (e.g., PI3K, Beclin-1), autophagosome formation and elongation (e.g., ATG5, ATG12, ATG16), and lipid conjugation (e.g., LC3, ATG3) (Mizushima et al. 2011). Other proteins can modulate autophagy activity. ULK2 is necessary for autophagy induction (Mizushima 2010); BCL2 inhibits Beclin-1-dependent autophagy (Pattingre et al. 2005); ADNP encodes a binding partner of LC3 (Gozes and Ivashko-Pachima 2015); and expression of p62, which directly interacts with LC3-I and LC3-II, is negatively related to autophagy (Pankiv et al. 2007).

13.4.1 Association with the Brodmann Area 22

The Brodmann area 22 (BA22) is part of the superior temporal gyrus that includes the well-known Wernicke's area playing critical roles in language processing. Both self-consciousness and language are regarded as the higher cognitive functions of the human brain.

The first line of evidence revealing a possible association between autophagy and SCZ was raised from a comparison of gene expression across multiple brain areas between postmortem SCZ patients and controls (Horesh et al. 2011). Surprising that a highly significant difference in gene expression was found in BA22, which is well known to be associated with positive symptoms of SCZ such as hallucinations and hearing voices that do not exist. It is notable that this difference in BA22 presents a cluster of ATGs and their expression levels are significantly decreased in SCZ patients. These ATGs include BECN1, ULK2, ATG3, which are related to the key steps (signaling, autophagosome formation, and lipid modification) of macroautophagy (Kroemer et al. 2010) (Table 13.1). This is an exciting finding because BA22 is critical for language processing, implying that hallucination in SCZ is associated with higher cognitive function. However, a limitation of this study is it used postmortem samples from SCZ patients who would have taken medications for many years. Thus, another possibility that cannot be ruled out is that the decreased expression of ATGs may be a result of the treatment because medications used to block D2 receptors in the BA22 and auditory–visual association cortices may be causing this effect as part of the drugs' action in controlling or suppressing hallucinations (Goldsmith et al. 1997).

The second study performed a similar analysis of gene expression in the postmortem samples from 23 SCZ patients compared with 19 controls, and it focused on the brain region BA22, which is implicated in positive symptoms, and BA10, which is implicated in negative symptoms of SCZ (Barnes et al. 2011). Consistent with the above finding, autophagy dysfunction was found to be more significant in BA22 than in BA10. Thus, these two studies have established a new connection between the expression of ATGs and SCZ (Schneider et al. 2016).

Nevertheless, these early findings needed to be further examined to determine whether autophagy dysfunction in BA22 is inheritable and to determine if there is concordance of autophagy dysfunction in BA22 between identical twins. Caution should be used because another postmortem study did not find any change of ATGs expression in BA22 from SCZ (Sellmann et al. 2014). Furthermore, brain imaging studies may be able to detect activity differences of BA22 between SCZ and healthy controls. It is a fact that the volume of the left anterior BA22 is smaller in SCZ than in controls, and it is negatively correlated with the severity of hallucinations. Additionally, the left BA22 is smaller than the right BA22, which is not observed in controls (Rajarethinam RP et al. 2000). These structural abnormalities of BA22 in SCZ have been confirmed by other brain imaging studies (Kasai et al. 2003; Sun et al. 2009; Penner et al. 2016). Furthermore, DARPP-32 (Dopamine and a cAMP-regulated phosphoprotein of molecular weight 32 kDa) is associated with a change

Table 13.1 Autophagy-related genes associated with SCZ

Gene	Function	BA22	Hipp	Peripheral blood	Circulating lymphocyte	References
ATG3	Participate in autophagy catalytic process	↓	↓			Horesh et al. (2011), Schneider et al. (2016) and Kroemer et al. (2010)
ADNP	Activity-dependent neuroprotective protein	↓	↓	↑	↑	Sragovich et al. (2017), Gozes and Ivashko-Pachima (2015) and Merenlender-Wagner et al. (2015)
ADNP2	ADNP2 (ADNP Homeobox 2) is a protein coding gene. An important paralog of this gene is ADNP	↓	↓	↑	↑	Sragovich et al. (2017) and Merenlender-Wagner et al. (2015)
Beclin-1	As a scaffolding protein assembling Beclin 1 interactome to regulate Class III PI3K/VPS34 activity, which in turn, tightly controls autophagy at multiple stages	↓	↓↓	–	–	Merenlender-Wagner et al. (2015), Schneider et al. (2016) and Kroemer et al. (2010)
Bcl2	Regulators of the cellular life-or-death switch, Encodes an integral outer mitochondrial membrane protein that blocks the apoptotic death of some cells such as lymphocytes	↓	↓	↑	↑	Horesh et al. (2011) and Merenlender-Wagner et al. (2015)
PI3KR4	Involved in cellular functions such as cell growth, proliferation, differentiation, motility	↓				Rapoport et al. (1999), Kroemer et al. (2010) and Schneider et al., (2016)
ULK2	Involved in autophagy in response to starvation. Part of regulatory feedback loops in autophagy	↓				Sumitomo et al. (2018), Schneider et al. (2016) and Kroemer et al. (2010)

(continued)

Table 13.1 (continued)

Gene	Function	BA22	Hipp	Peripheral blood	Circulating lymphocyte	References
DARPP-32	Key downstream effector in transducing dopamine signaling, integrating the pathways of different neurotransmitters and neuromodulators	↓	–			Albert et al. (2002), Ishikawa et al. (2007) and Kunii et al. (2014)
p-DARPP-32	DARPP-32 phosphorylation	↓	–			Kunii et al. (2011, 2014)

↓ mRNA decrease $p < 0.05$ versus Control
↓↓ Significantly decrease $p < 0.01$
↑ mRNA Increase
– No differences
Hipp, Hippocampus

of dopamine and glutamate systems in the pathogenesis of SCZ (Greengard et al. 1999). Kunii Y et al. reported that DARPP-32 and phosphorylated DARPP-32 were both decreased in BA22 in 11 SCZ patients compared with 11 age- and sex-matched controls (Kunii et al. 2011) (Table 13.1). BA22 is also associated with other cognitive functions including social communication and interaction, which are also impaired in SCZ. Based on these lines of evidence, we suggest that exploring the connection between the expression of ATGs and SCZ in BA22 may reveal a new understanding of the neuropathology of SCZ.

13.4.2 Association with the Hippocampus

The hippocampus is part of the allocortex, located under the cerebral neocortex, and it plays critical roles in memory formation, spatial navigation, stress response, etc.

Merenlender-Wagner et al. reported an interesting finding supporting the connection between the hippocampus and autophagy in SCZ (Merenlender-Wagner et al. 2015). The authors found that the expression of Beclin-1 mRNA was decreased in the hippocampus but not in the lymphocytes of SCZ patients. In marked contrast, Bcl2, ADNP, and ADNP2 mRNA were significantly increased in the lymphocytes of SCZ patients (Table 13.1). An animal study further found that ADNP2 but not ADNP was increased as a result of chronic clozapine treatment. Thus, it is possible that Bcl2, a BECN1-interacting antiapoptotic protein, and ADNP-LC3B interactions may represent biomarkers for SCZ onset. Consistent with this finding, brain imaging

studies have found a reduction of gray matter volume in the CA2 of the hippocampus in SCZ patients (Benes et al. 1998; Glantz et al. 2006). The function of CA2 is not fully understood yet, but evidence has suggested that CA2 is critical for social memory (Hitti and Siegelbaum 2014). This line of evidence implies that autophagy dysfunction in the hippocampus, especially in the CA2 region, may be related to the deficits of social communication and interaction in SCZ patients.

It is notable that one of the most consistent findings in SCZ is an abnormality of the hippocampal structure and function (Bogerts et al. 1985; Nelson et al. 1998; Benes et al. 1998; Heckers and Konradi 2002). The first study on hippocampal volume in SCZ patients relative to controls was performed by Bogerts et al. in 1985, who reported a 40% difference of the hippocampal volume in SCZ patients. A meta-analysis (Nelson et al. 1998) has concluded that the hippocampal volume reduction is merely 5% in SCZ, but this is still the most robust and replicated finding in the SCZ research fields. Similar to the risk for the development of posttraumatic stress disorder (PTSD), a smaller hippocampal volume can predict a higher risk of children for developing SCZ as an adult (Lawrie et al. 1999; Copolov et al. 2000; Pantelis et al. 2003).

13.4.3 No Association with the Brodmann Area 10

The Brodmann area 10 (BA10) is a part of the prefrontal cortex located in the frontal pole. It is involved in cognitive functions such as memory recall and various executive functions, attention, decision making, etc.

It is very interesting that a connection between ATGs expression and SCZ was not found in BA10 (Horesh et al. 2011; Barnes et al. 2011; Maycox et al. 2009). In marked contrast, the expression of many genes including DISC1, dysbindin, CAMKK2, etc., are changed in BA10. This finding implies that autophagy may contribute to certain aspects of SCZ, mainly related to functions of the BA22 and the hippocampus. It would be interesting to know the details of the symptoms of the SCZ patients who donated the postmortem samples because that information would be valuable for analyzing the association between the BA22- and the hippocampus-associated symptoms and ATGs expression.

13.5 The Causality Between ATGs and SCZ

In the field of psychiatric disorders, the association among different factors such as that between ATGs expression and SCZ is often not solid as it is often not replicable across studies on different populations. To understand whether ATGs could contribute to the neuropathology of SCZ, the causality between autophagy dysfunctions and SCZ should be further clarified. Thus, animal models of SCZ are necessary for this purpose.

The ATG ULK2 is essential for autophagy induction (Mizushima 2010), leading to changes of p62 and GABAA receptor trafficking and unbalance of excitation and inhibition in the prefrontal cortex. In the Ulk2 heterozygous mice, expression of p62, an autophagy-associated stress response protein, was increased predominantly in pyramidal neurons of the medial prefrontal cortex (mPFC). These mice exhibited deficits of sensorimotor gating and cognition. In contrast, the surface expression of GABAA receptors in pyramidal neurons of the mPFC was decreased. Since copy number variations of Ulk2 have been found in SCZ patients, this study supports the hypothesis of the inheritability of autophagy-related mechanisms since artificially manipulated Ulk2 heterozygosity is likely to be at least partially responsible for the neuropathogenesis of SCZ (Sumitomo et al. 2018). A similar mechanism of Ulk2 resulting in unbalanced excitation and inhibition could occur in BA22 because Ulk2 mRNA has also been found to be decreased in postmortem samples of BA22 from SCZ patients (Schneider et al. 2016). In short, autophagy failure due to Ulk2 deficiency can lead to certain symptoms of SCZ.

The ATG BECN1 (Beclin-1) interacts with either Bcl-2 or PI3k class III, playing a critical role in the regulation of both autophagy and cell death. Thus, it is not surprising that decreased Beclin-1 expression in the hippocampus and the prefrontal cortex is associated with SCZ (Merenlender-Wagner et al. 2015), MDD, and Alzheimer's disease (AD), etc. It is known that increased levels of Beclin-1 lead to autophagy but decreased levels of Beclin-1 cause apoptosis. Animal studies have found that the administration of NAP, a peptide fragment of ADNP, can enhance ADNP-LC3 interactions, leading to a reversal of decreased Beclin-1 mRNA in the hippocampus in a mouse model of SCZ (MAP6-deficient mouse). The rescued Beclin-1 expression by NAP also led to positive behavioral changes in the mice (Merenlender-Wagner et al. 2014). This study's results suggest that decreased Beclin-1 mRNA may cause SCZ-like behaviors but an increase of Beclin-1 expression by NAP can reduce SCZ-like behaviors, relevant to the development of a novel therapy by enhancing autophagy to treat SCZ. In other words, the ATGs BECN1 expression seems to be both necessary and sufficient for the neuropathogenesis of SCZ.

The ATGs ADNP (activity-dependent neuroprotective protein) and ADNP2 encode the proteins interacting with LC3, a critical component of the autophagosome (Gozes and Ivashko-Pachima 2015). However, ADNP is not unique for autophagy in SCZ but is also involved in autism. Interesting, ADNP2 expression is significantly increased by clozapine treatment. Since ADNP heterozygous can lead to cognitive deficits and social dysfunction (Vulih-Shultzman et al. 2007), the interaction with ADNP–LC3B may be required for an autophagy process that is linked with SCZ-like behaviors (Merenlender-Wagner et al. 2015).

The expression levels of ADNP and ADNP2 have been found to be highly correlated in the hippocampus and in the prefrontal cortex of postmortem SCZ samples. It is assumed that the ADNP/ADNP2 expression may relate to the progression of SCZ (Sragovich et al. 2017).

13.6 mTOR Regulation of Autophagy

The mammalian target of rapamycin (mTOR) is a member of the phosphatidylinositol kinase-related kinase (PIKK) family, and it is important in regulating the cell cycle (Heitman et al. 1991). Nutrient starvation induces autophagy through inhibition of mTOR. mTOR binds with several proteins to form two distinct protein complexes: mTORC1 contains raptor, GβL/mLst8, PRAS40, and DEPTOR; mTORC2 contains RICTOR, GβL/mLst8, Sin1, PRR5/protor and DEPTOR (Hara et al. 2002; Kim et al. 2002).

mTORC1 is a key mediator from growth factor signaling to autophagy. The insulin/insulin-like growth factor (IGF-1) pathway involves PDK1 and Rheb and upregulates mTORC1 signaling. Conversely, PTEN and TSC2 downregulate mTORC1 signaling. Cellular stresses such as hypoxia and starvation induce autophagy in a manner dependent on mTORC1. mTORC1 is found in the proximity of mitochondria, and it is inhibited by oxidative stress and mitochondrial dysfunction, also implicating that mTORC1 is involved in mitophagy. Mitophagy promotes turnover of mitochondria and prevents the accumulation of dysfunctional mitochondria, which can lead to neuronal degeneration. This process is regulated by PINK1 and parkin that are suggested to be associated with the neuropathogenesis of Parkinson's disease (PD).

It is not clear yet how mTORC2 is activated, but it plays an important role in Akt phosphorylation that activates mTORC1 (Jacinto et al. 2006). Other substrates of mTORC2 are protein kinase Cα, playing roles in synaptic plasticity, actin dynamics, and neuronal morphology (Sarbassov et al. 2004), which are the fundamental basis of cognitive functions.

If there is an imbalance between synthesis and degradation of proteins involved in SCZ, would not only autophagy but also mTOR signaling equally contribute to SCZ? In fact, there is a wealth of evidence supporting the connection between mTOR signaling and SCZ (e.g., see the review by (Gururajan and Van den Buuse 2014). Because mTOR signaling-dependent protein synthesis is linked with SCZ, it is easier to understand why degradation of proteins by autophagy is also regulated by mTOR signaling, and easier to understand the underlying mechanisms for the balance between synthesis and degradation of proteins.

Many studies have consistently reported that mTORC1 can phosphorylate Atg13 and ULK1/2. In contrast, inhibition of mTORC1 by rapamycin or starvation leads to dephosphorylation of ULK1/2 and Atg13 (Jung et al. 2009; Ganley et al. 2009; Hosokawa et al. 2009). Furthermore, FIP200 phosphorylation is inversely correlated with mTORC1 activation, implying that ULK1/2 could phosphorylate FIP200. Another study found that FIP200 binds to ULK via Atg13 to be phosphorylated (Jung et al. 2009).

We are not going to further discuss the regulation of mTOR signaling on autophagy (see review by Jung et al. 2010). The conditions of poor nutrients, growth factor signaling and stress response pathways induce autophagy via interactions between mTORC1 activation and FIP200/Atg13/ULK complexes. However, it is notable that

mTOR signaling is not necessary for supporting the association with autophagy because it plays general roles in regulating cell cycle and protein synthesis.

13.7 SCZ Medication Regulation of Autophagy

The first-generation of typical antipsychotic agents such as fluspirilene, trifluoperazine, and pimozide are not particularly selective and just block dopamine receptors to produce a neuroleptic effect. These drugs are also used to treat Huntington's disease, and all of them induce autophagy in human cells in vitro by increasing LC3-II expression (Zhang et al. 2007). However, atypical antipsychotics such as clozapine are reported to block autophagy by inhibiting the formation of autophagolysosomes in rat primary neurons (Park et al. 2012), but they also enhance ULK1–Beclin-1 signaling in the rat frontal cortex (Kim et al. 2018; Vucicevic et al. 2011). Most of the antipsychotics increase LC3-II expression via different mechanisms of autophagy (Vucicevic et al. 2018). However, caution still needs to be applied when considering whether inducing autophagy can produce antipsychotic effects.

13.8 Conclusion

Autophagy and its related mechanisms provide a new understanding of the neuropathogenesis of SCZ. The strongest line of evidence came from the expression of ATGs in the BA22 of postmortem samples from SCZ patients, which is in agreement with some brain imaging studies and certain hypotheses of SCZ in interpreting its positive symptoms. Nevertheless, other studies using a similar strategy did not find an association with ATGs expression, just with genes that are well-known to be associated with SCZ. mTOR regulation of autophagy is potentially a piece of strong supporting evidence for autophagic neuropathogenesis of SCZ, considering the balance between synthesis and degradation of proteins essential for maintaining basic physiological conditions of neurons. However, there is still a lack of direct evidence supporting the hypothesis that mTOR signaling-dependent autophagy is both necessary and sufficient for the neuropathogenesis of SCZ. The hypothesis is supported by the finding that antipsychotics often induce autophagy in data from in vitro studies through distinct mechanisms of drug action, which may all share as common features both autophagy induction and antagonist action against dopamine receptors. It is thus worthy of further study to determine how antagonism of dopamine receptors may contribute to inducing autophagy or whether enhancing autophagy may work as augmentation for enhancing the effects of antipsychotics. Most importantly, since some factors related to autophagy can be detected in lymphocytes and they are sensitive to drug treatment, it is possible that detecting autophagy and related mechanisms may potentially serve as biomarkers for diagnosis and prognosis. This direction following autophagy and its related mechanisms seems very promising.

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Part II
Autophagy and Cardiovascular Diseases

With the rapid economic development in China, the incidence and mortality rate of cardiovascular diseases (mainly hypertension, coronary heart disease, and stroke) have gradually been increasing yearly since the 1990s, and the onset age has decreased. A recent 8-year follow-up study indicated that among nearly 170,000 people older than 40 years in China, the top three causes of death were heart diseases (296.3), malignant tumors (293.3), and cerebrovascular diseases (276.9) (mortality rate/100,000 people per year). Heart diseases account for 22.5% of the total deaths, topping the list, and have become a serious disease threatening the health of old, middle-aged, and young people in China.

Autophagy is a degradation process that depends on lysosomes characterized by cytoplasmic vacuolation. This process is a universal phenomenon specific to eukaryotes. Autophagy degrades damaged cell plasma membranes and organelles via lysosomes. The degraded products can be used in energy generation and the synthesis of proteins and plasma membranes to support cell metabolism and the renewal of senescent damaged cellular constituents, thus maintaining cell survival, differentiation and development, and a stable internal environment. Autophagy participates in multiple physiological and pathological processes. This process has been shown to participate in the regulation of the steady state in the cardiovascular system, playing important roles in the physiopathological processes of multiple cardiac diseases (including cardiomyopathy, cardiac hypertrophy, ischemic heart disease, and heart failure). Appropriate autophagy can maintain cardiovascular stability. However, insufficient autophagy or excessive autophagy may promote cardiovascular diseases or aggravate lesions.

Chapter 14
Autophagy and Hypertension

Jie Du, Congcong Zhang, and Wei Zhao

Abstract Hypertension is one of the most common cardiovascular diseases. Sympathetic hyperactivity and renin–angiotensin–aldosterone system activation cause changes in cardiovascular structure and function. Autophagy plays an important role in cellular waste removal and structural reconstruction. In this chapter, we will introduce the role of autophagy in the pathogenesis of hypertension and the resulting heart damage.

Keywords Autophagy · Hypertension · Angiotensin II · Inflammation

Hypertension is a systemic disease characterized by persistently elevated blood pressure during the systolic and/or diastolic phase in the systemic circulation and is one of the most important risk factors for cardiovascular diseases. In China, elevated blood pressure has a persistent positive correlation with the onset of cardiovascular diseases. Systolic pressure decreased by 10–12 mmHg or diastolic pressure decreased by 5–6 mmHg can reduce the incidence of stroke by 35–40%, that of myocardial infarction by 20–25% and that of heart failure by 50%. Hypertension directly results in damage to multiple target organs, such as the heart, brain, and kidney.

The pathogenesis of hypertension includes the following:

1. Hyperfunction of sympathetic nerve activity, which plays a very important role in the formation and maintenance of hypertension. Approximately 40% of primary hypertension patients have an elevated blood catecholamine level. Long-term activation of a sympathetic nerve may lead to increased secretion of peripheral catecholamine, arteriole, and vein contraction, and increased cardiac output. It may also influence renal blood flow and cause sodium water retention, resulting in further elevated blood pressure.
2. Activation of the renin–angiotensin–aldosterone system (RAAS), which is closely associated with injury of target organs caused by hypertension development. Renin, secreted by glomerular juxtaglomerular cells, activates hepatocytes to generate angiotensinogen, and then angiotensin (Ang) I. Next, this molecule

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is hydrolyzed into Ang II by pulmonary vascular endothelial cells. Within a short time, Ang II acts on the Ang I receptor in the heart and blood vessels, contracts small vessels, stimulates the secretion of aldosterone to increase the blood volume, and promotes the secretion of catecholamine to elevate blood pressure. Long-term Ang II accumulation in the tissue leads to myocardial hypertrophy and cardiac interstitial fibrosis remodeling, resulting in kidney and brain injury as well as vascular remodeling.

3. Multiple factors, such as RAAS, inflammation, and oxidative stress, cause endothelial dysfunction and disorder in vasomotion.

The above factors are the important reasons for injury of target organs and the complications caused by hypertension. In the above pathological processes, pressure and volume load lead to increased mechanical stress of the vascular wall, which directly induces cell autophagy to maintain stability of the intracellular environment. Weibel–Palade bodies (WPB) in endothelial cells rapidly release bioactive molecules and regulate endothelial function upon stimulation. WPB are located in autophagosomes. An autophagy inhibitor or knockdown of autophagy-related (ATG) 5/7 significantly inhibited the secretion of bioactive molecules by WPB, leading to endothelial cell dysfunction. In senescent endothelial cells, the expression of ATGs was significantly reduced, accompanied by decreased nitric oxide (NO) synthesis, increased oxidative stress and the generation of inflammatory factors. Inhibiting autophagy can inhibit endothelial diastolic function. In contrast, an autophagy inhibitor suppressed the generation of reactive oxygen species (ROS) and inflammatory factors, and thereby recovered the diastolic function of endothelial cells. Prorenin receptor (PRR), which was discovered in 2002, has a high affinity for renin and prorenin. Specific overexpression of PRR in smooth muscle cells led to elevated blood pressure. PRR catalyzes the transformation of angiotensinogen into Ang I, and its intracellular fragment participates in the mTOR-autophagy signaling pathway, suggesting that autophagy is associated with the pathogenesis of hypertension. Ectopic extracellular matrix (ECM) deposition in blood vessels occurs during hypertension. ECM is a critical regulatory factor for the survival, proliferation and migration of endothelial cells. Decorin (DCN) is a secreted matrix proteoglycan. Soluble DCN treatment of mouse endothelial cells significantly increased the formation of autophagosomes, which depends on the interaction between DCN and vascular endothelial growth factor receptor 2 (VEGF2/KDR). DCN can enhance the expression of paternally expressed 3 (PEG3). PEG3 binds to the positive phagocytic complex of Beclin 1 and LC3 and further fuses with a multimolecular complex containing Beclin 1 and LC3, increasing autophagic flux. Various endothelial vascular constriction factors and relaxing factors secreted by endothelial cells act on smooth muscle cells to regulate vascular tension and blood pressure. Thus, smooth muscle cells play a very important role in maintaining blood pressure. Platelet-derived growth factor (PDGF) can induce autophagy of smooth muscle cells. Inhibition of autophagy can inhibit the phenotype transformation of smooth muscle cells induced by PDGF.

Left ventricular hypertrophy is another common complication of hypertension. Currently, the reasons for the formation of myocardial hypertrophy mainly include

hemodynamics, neurological endocrine activity, and immune inflammation. Pressure and/or overload can increase ventricular wall tension, activate the neuropathic endocrine-inflammatory factor system, initiate the hypertrophy signaling pathway, lead to activation and expression of multiple transcription factors and embryonic genes (such as β-myosin), increase the RNA and protein synthesis rates, and promote cardiomyocyte hypertrophy. Protein renewal in hypertrophic cardiomyocytes is accelerated, as well as autophagy. The pathological factors promoting myocardial hypertrophy (such as excessive high tension of cardiac sympathetic nerves, oversecretion of isoprenaline, and increased cardiac afterload) inhibit the occurrence of cardiomyocyte autophagy. Specific knockout of ATG5 in cardiomyocytes may inhibit the occurrence of myocardial hypertrophy and heart failure caused by autophagy in cardiomyocytes. Cardiomyocytes from ATG5-knockout rats were more sensitive to isoprenaline than their wild-type counterparts. Under isoprenaline stimulation, cardiac function was significantly reduced, suggesting that autophagy can counteract the negative effects of epinephrine. Rapamycin inhibited myocardial hypertrophy caused by ascending aorta ligation, myocardial infarction, and thyroid hormone by activating autophagy. These studies indicated that autophagy in cardiomyocytes can protect the body from myocardial hypertrophy caused by adrenaline or pressure overload. In contrast, a histone deacetylase inhibitor was reported to inhibit pathological myocardial hypertrophy by inhibiting autophagy. Ang II maintains blood volume, regulates blood pressure, oxidative stress and the inflammatory response, and improves the synthesis of myocardial proteins, leading to myocardial hypertrophy and cardiac function injury. Ang II was upregulated in the heart of hypertension patients, and autophagic activity was enhanced simultaneously, suggesting the existence of a mechanism involving Ang II and autophagy. Ang II stimulated cardiomyocytes with overexpression of Ang II receptor type 1 (AT_1) receptors to activate autophagy but stimulated those overexpressing AT_2 receptors to inhibit autophagy, suggesting that Ang II dually regulates autophagy in cardiomyocytes via AT_1/AT_2 receptors (Porrello et al. 2009; Porrello and Delbridge 2009). Thus, the different effects of autophagy in myocardial hypertrophy may be caused by pathological stimulation, degree of disease injury, autophagy level, and occurrence stage.

Inflammation is a critical process in target organ injury induced by hypertension. Macrophages are important cells mediating the inflammatory response. These cells participate in cardiac inflammatory injury, myocardial hypertrophy, and cardiac fibrosis. Multiple stimuli can induce monocytes to pass through the vascular wall from the circulation into the injured site in the heart and differentiate into macrophages. Inflammatory cytokines are generated after macrophage activation, leading to the activation of myofibroblasts and cardiomyocyte hypertrophy (Jia et al. 2012). The autophagy pathway plays a very important role in regulating immune inflammation. The mitochondrion is the main organelle generating free radicals during oxidative phosphorylation and is the main target of oxidative damage during stress. Ang II has been proven to stimulate ROS generation in the mitochondria of cardiomyocytes, leading to reduced potential of the mitochondrial membrane, increased oxidative

stress damage to mitochondrial proteins and promotion of mitochondrial DNA deletion. Ang II can also increase autophagosome generation in mitochondria to scavenge ROS, promote mitochondria biosynthesis, supplement injured mitochondrial proteins and recover energy generation. If cathepsin S is missing, autophagic vacuoles in mitochondria cannot bind to lysosomes, resulting in increased accumulation of autophagic vacuoles, disordered autophagic function, and further increased ROS accumulation, activating the NF-κB inflammatory signaling pathway, promoting cardiac fibrosis, and aggravating cardiac trauma induced by Ang II perfusion. Ang II has been shown to upregulate ATG5 expression in macrophages. ATG5 regulates autophagy in mitochondria, scavenges ROS, and alleviates the inflammatory response. Decreased ATG5 expressions in macrophage downregulate mitochondrial autophagy. ROS accumulation activated the NF-κB inflammatory signaling pathway, promoted proinflammatory factor secretion, and aggravated cardiac inflammation and injury induced by Ang II perfusion (Zhao et al. 2014).

Currently, antihypertensive therapy is the main strategy for treating hypertension, assisted by various non-pahrmacological treatments simultaneously, primarily including quitting smoking and drinking and weight loss by exercising. Antihypertensive drugs are the main drugs used in this treatment, aiming to adjust blood pressure to a normal level, especially for patients with metabolic syndrome, type 2 diabetes or insulin resistance. Hypertension is a chronic disease that requires long-term medication to maintain antihypertensive efficacy. However, the risk of hypertension for patients with other cardiovascular diseases is higher than that for healthy people. Therefore, new therapeutic methods and targets are still needed. Currently, anti-inflammatory and other biological treatments are being researched with a wide development range. Autophagy participates in multiple pathological processes linked to hypertension and target organ injury. A deep understanding of the pathological mechanism of autophagy that regulates hypertension, especially target organ injury caused by terminal stage hypertension, may provide new clues and targets for hypertension prevention and treatment.

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Chapter 15
Autophagy and Myocardial Ischemia

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Abstract Myocardial ischemia and reperfusion cause injury to the heart in myocardial ischemic disease. Both processes increase autophagy. In this chapter, we will provide an overview of the autophagic mechanism caused by myocardial ischemia/reperfusion injury and the role of autophagy in myocardial ischemia/reperfusion injury.

Keywords Autophagy · Myocardial ischemia · Ischemia/reperfusion · Energy metabolism · Reactive oxygen species

Coronary artery disease arises from luminal stenosis or occlusion caused by atherosclerosis lesions in the coronary artery, resulting in myocardial ischemia, hypoxia, or necrosis. Based on severity, this condition is divided into five clinical types: asymptomatic myocardial ischemia (occult coronary artery disease), angina pectoris, myocardial infarction, ischemic heart failure (ischemic heart disease), and sudden death. The total number of patients with coronary artery diseases in China is approximately 130,000,000, and approximately 1,000,000 people die of various coronary heart diseases every year. Therefore, coronary artery disease is the most common disease severely threatening people's health. Most patients die of acute myocardial infarction or sudden death. The main reasons are myocardial ischemic necrosis and malignant arrhythmia or cardiogenic shock after complete obstruction of the coronary artery caused by coronary vasospasm or intravascular atherosclerotic plaque rupture due to tiredness, stress, and smoking. The main symptom of myocardial infarction is typical chest pain in the retrosternal or precordial region, accompanied by pyrexia, diaphoresis, panic, nausea, and vomiting. Approximately one-third of patients show sudden death in the first onset. The current main treatment is to alleviate stenocardia symptoms, quickly recover the blocked blood flow, and reduce the myocardial infarction range and long-term mortality rate.

At the time of myocardial infarction, the coronary artery participating in supplying nutrients and oxygen to the myocardium is occluded; hence, necessary molecules for cell survival are lacking. When the artery is reopened, the surviving

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cells become injured by the sudden increase in oxygen. The whole process is called ischemia/reperfusion (IR), which induces a series of expression changes in proteins and genes associated with death promotion and survival inhibition. Because the cells are injured, the contraction capability of the heart is reduced, accompanied by post-injury remodeling. Early manifestations of cardiac remodeling include ventricular dilatation, cardiomyocyte hypertrophy, increased cytokine levels, and autorhythmicity changes in cardiomyocytes, thus maintaining cardiac function. Manifestations at late remodeling include fibrosis, collagen deposition, calcium flow disorder, thinner ventricular walls, and persistently increased cell death rate, finally leading to heart failure. The death of cardiomyocytes under ischemia is an initiating factor for cardiac remodeling; thus, exploration of protective mechanisms for avoiding cardiomyocyte death is necessary.

In theory, under ischemia and hypoxia, cardiomyocytes die via apoptosis or necrosis. In-depth studies have revealed that autophagy can regulate the survival of cardiomyocytes under ischemia and hypoxia.

15.1 Reasons for Autophagy Induced by IR

Recently, multiple studies have demonstrated that the number of autophagosomes formed in the myocardial ischemic stage or reperfusion stage is significantly increased. Sybers et al. found that autophagosomes were generated in the mouse embryonic heart under reoxygenation conditions after hypoxia and glucose deprivation (Sybers et al. 1976). Decker and Gurusamy showed that ischemia for 20 min alone could not induce autophagy in the Langendorff heart IR model, but ischemia for 40 min induced the formation of autophagosomes. After reperfusion, the number of autophagosomes was further increased, and the expression of autophagy-related genes (LC3-II and Beclin 1) was significantly increased (Decker and Wildenthal 1980; Gurusamy and Das 2009). Furthermore, autophagy was found to be increased after chronic ischemia in human and pig heart tissues, and the formation of autophagosomes and increased lysosomal activity was observed in rat and mouse hearts after acute IR. In the myocardial infarction model induced by left coronary artery ligation, autophagosome markers (LC3 and p62) were increased, and many autophagosomes were generated in the infarction margin.

The mechanisms causing autophagy during IR include:

(1) Change of ATP level and activation of the AMP-activated protein kinase (AMPK) signaling pathway in the myocardial ischemic stage: during in vitro culture, deprivation of glucose can reduce the intracellular ATP level, which is consistent with upregulation of autophagy. In vivo, the intracellular ATP content is insufficient, and the AMP and ATP ratios increase to activate AMPK at the time of myocardial ischemia. Activated AMPK induces autophagy by indirectly or directly modifying Unc-51-like autophagy activating kinase 1 (ULK1). Matsui et al. found that glucose deprivation in cardiomyocytes cultured in vitro led

to autophagosome formation by activating AMPK and negatively regulating the mTOR signaling pathway (inhibitor of autophagy) (Matsui et al. 2007). AMPK inhibits mTORC1 by phosphorylating TSC2 and Raptor and then indirectly activates ULK1 (Alers et al. 2012). Recently, AMPK was shown to directly phosphorylate and activate ULK1, thus inducing autophagy. When the activity of AMPK is inhibited, the autophagy induced by myocardial ischemia is reduced.

(2) SIRT1–FOXO-dependent mechanism: Sebastiano et al. found that under starvation, SIRT1 mediates the deacetylation of FOXO, increases the expression of the GTP-binding protein Rab7, mediates autophagosome-lysosome fusion and enhances autophagy. When FOXO1 is downregulated or deacetylation is inhibited, autophagy is attenuated. The abovementioned studies indicate that myocardial ischemia may regulate autophagy via a SIRT1–FOXO-dependent mechanism.

(3) The BNIP3 pathway (a proapoptotic protein Bc122 family member): Zhang et al. revealed that during myocardial hypoxia, BNIP3 mediated the upregulation of hypoxia-inducible factor 1 and activated autophagy. After siRNA silencing of BNIP3, hypoxia-induced autophagy was attenuated, indicating that BNIP3 also participates in the regulation of autophagy at the time of myocardial hypoxia (Azad et al. 2008).

(4) Calcium overload in mitochondria: the Ca^{2+} concentration in ischemic cardiomyocytes significantly increases with the opening of sodium–calcium exchanger calcium channels and becomes an inducing factor of autophagy. Ca^{2+} mobilization agents, such as vitamin D, ionomycin, ATP, and carotene, can inhibit the mTOR signaling pathway, leading to the accumulation of Beclin 1- and ATG7-dependent autophagosomes.

(5) ROS: IR increases the intracellular ROS level. However, an in vitro study indicated that LPS promotes the generation of ROS in cardiomyocytes of suckling mice and induces autophagy formation. ROS scavengers could reduce this formation (Hickson-Bick et al. 2008). Autophagosome formation is inhibited by oxidation of the serine subunit of ATG4 by hydrogen peroxide, which is generated during starvation, thereby deactivating autophagy.

(6) Endoplasmic reticulum (ER) stress and the unfolded protein response: ER stress and the unfolded protein response in cardiomyocytes of IR and myocardial infarction model in mice are activated. Tunicamycin and carotene (two activators of ER stress) can induce autophagosome formation. As an effective molecule of ER stress, IRE1 is necessary for autophagosome formation. All these investigations suggest that ER stress is a regulatory mechanism formed by autophagy in ischemic cardiomyocytes.

(7) Opening of mitochondrial permeability transition pores (MPTPs): MPTPs on the mitochondrial membrane are closed under physiological conditions and open when cells are stimulated. MPTP opening during IR promotes cell autophagy. Cyclosporine or sanglifehrin-A can inhibit cardiomyocyte autophagy in the reperfusion stage.

15.2 Protective Effects of Autophagy in IR Injury

15.2.1 Maintenance of ATP Balance

The basic function of autophagy is degradation. In autolysosomes, proteins and cell membranes are degraded, and free fatty acids and amino acids are released as raw materials to synthesize ATP in the tricarboxylic acid cycle. Matsui et al. showed that glucose deprivation reduces the intracellular ATP level (Matsui et al. 2007), which is associated with the upregulation of autophagy. The autophagy inhibitor 3-methyladenine (3-MA) further reduces the ATP concentration and increases the mortality rate of cells. This finding suggests that autophagy activated during the ischemic process can promote cell survival by maintaining the ATP concentration.

15.2.2 Alleviation of Mitochondrial Autophagy in Mitochondrial Injury

Mitochondria in ischemic cardiomyocytes are damaged because of energy deficiency. The damaged mitochondria can generate excessive amounts of ROS and further consume ATP. Moreover, the damaged mitochondria release cytochrome C, apoptosis-inducing factor (AIF) and other proapoptotic factors, thus damaging more mitochondria. Scavenging the damaged mitochondria is a protective effect of cells. Autophagy is the only way to degrade organelles. Scanning electron microscopy has shown that the autophagosomes formed during IR contain mitochondria. The numbers of mitochondria and sarcomeres were increased in cardiomyocytes of mice with *Atg5* gene deletion. Mitochondrial lysis is an essential condition for mitophagy. In the myocardium after IR, chondriokinesis occurs earlier than autophagosome formation. Furthermore, chondriokinesis inhibition can reduce autophagosome formation in ischemic myocardium.

15.2.3 Protein Scavenging

During ischemic hypoxic injury in cardiomyocytes, high levels of unfolded proteins are observed in the cells. Scavenging of these proteins requires autophagy and the ubiquitin protease system. Rapamycin-activated autophagy was shown to promote scavenging of intracellular aggregated proteins and reduce protein aggregation. While proteasome activity is inhibited, autophagy inhibition will increase the aggregation of intracellular proteins. Therefore, autophagy scavenges proteins and alleviates ER stress caused by these proteins; thus, it has protective effects on ischemic myocardium.

15.3 Possible Mechanism of Autophagy Promotion in Cardiac IR Injury

In contrast, several studies have indicated that activated autophagy induced in the reperfusion stage promotes cell death. Zhai et al. found that during IR, excessive autophagy accelerated the degradation of organelles and proteins that inhibit cardiomyocyte death, leading to more death and aggravation of cardiac injury caused by myocardial infarction. During IR in *Beclin* $1^{+/-}$ mice, autophagy was reduced, as well as the myocardial infarction area; thus, cardiac function was improved. The results of this study indicated that during IR, myocardial injury induced by excessive autophagy can be attenuated by low temperatures and inhibition of oxidative stress. The uncertainty regarding autophagy during reperfusion could be interpreted as follows.

In the cardiac reperfusion stage, although the energy crisis (such as ATP) has been eased, other proautophagy mechanisms, such as oxidative stress, mitochondrial injury, ER stress, and the inflammatory response, can activate autophagy. Autophagy is a nonspecific degradation mechanism. Degradation of some key hydrolases leads to enhanced oxidative stress and promotes apoptosis. For example, during reperfusion, the generated ROS induce autophagy to degrade catalase, generating hydrogen peroxide. The elevated hydrogen peroxide level aggravates autophagy. Positive feedback between autophagy and oxidative stress leads to autophagic cell death. ROS can directly stimulate autophagosome formation by oxidative modification. For example, hydrogen peroxide oxidizes the cysteine residues of Atg4, inhibits the activity of cysteine proteases and stimulates starvation-induced autophagy. ROS can induce permeability changes in mitochondrial membranes and activate autophagy by mitochondrial injury. These findings indicate that autophagy protects cardiomyocytes, whereas other studies indicate that autophagy promotes cardiomyocyte apoptosis.

There are many linkages between autophagy and the apoptotic pathway: on one hand, some autophagy-related proteins simultaneously participate in the regulation of autophagy and apoptosis. For example, ATG5 can transfer into mitochondria, interact with Bcl-xl (an antiapoptotic molecule), and induce cytochrome C release and thus apoptosis. On the other hand, some antiapoptotic proteins simultaneously participate in the regulation of autophagy and apoptosis. For example, Bcl-2 affects autophagy. The proautophagy effect of Beclin 1 is often negatively regulated by Bcl-2, which interacts with it. During reperfusion, the increase in Bcl-2/Bcl-xl-binding proteins (such as BNIP3) and the decrease in Bcl-2 aggravate Beclin 1-induced cell apoptosis and autophagy. Elevated BNIP3 can destroy the integrity of mitochondria and increase the levels of hyperoxides and proapoptotic factors. Furthermore, there is a mutual effect between autophagy and apoptosis. For example, Beclin 1 possesses one BH3 structural domain that can bind to and inhibit the proapoptotic Bcl-xl, suggesting that Beclin 1 can directly activate apoptosis. When Beclin 1 is hydrolyzed by Calpain and the domain is exposed, Beclin 1 can be transferred to a proapoptotic protein beyond the protective capability of autophagy. ATG5 is upregulated during heart IR. ATG5 has been demonstrated to directly interact with the Fas-related protein

death domain and stimulate autophagic cell death. The above studies indicate that autophagy has dual effects on heart IR. The determining factor is still not clear, but it may be associated with the autophagy level and duration.

Autophagy induced by reperfusion injury may be directly related to the preceding ischemic degree. If it is mild, the enhancement of induced autophagy at the reperfusion stage is beneficial. In contrast, overactivation of autophagy will result in scavenging necessary proteins or organelles in the cells, leading to cell dysfunction and autophagic death. Furthermore, autophagy is associated with inducing factors, such as hypoxia and lack of glucose, which may lead to the opposite effects.

With continuous investigations of autophagy, selective regulation of autophagy may be a new therapeutic target for the prevention and treatment of cardiac IR. Because autophagy may have dual functions at the myocardial IR stage, it is very important to use autophagy as a therapeutic target to differentiate between its protective and aggravation effects on tissue injury. More in-depth investigations are needed to explore the autophagy-activated transduction pathway, activation degree, and correlation between time and cell survival via gene or drug intervention under different conditions.

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Chapter 16
Autophagy and Heart Failure

Jie Du, Yan Liu, and Jintao Fu

Abstract Pressure overload can lead to cardiac hypertrophy and heart failure. Autophagic activity could be detected in heart failure patients and animal models. However, the role of autophagy in heart failure is not clear. In this chapter, we will outline the role of macroautophagy and mitophagy in heart failure and the mechanisms underlying regulation.

Keywords Autophagy · Cardiac hypertrophy · Heart failure · Mitochondrial autophagy · microRNA

Heart failure causes gradually aggravated exertional or resting dyspnea in patients with coronary heart diseases, hypertension, diabetes, or myocarditis, after heavy drinking, or receiving chemotherapy and/or radiotherapy. This condition also results in lassitude, fluid overload (such as pulmonary edema or ankle edema), and abnormal cardiac structure and function. Heart failure is a major etiology of admitted people over 65 years of age. In developed countries, the average age of onset of heart failure is 75 years. In developing countries, 2–3% of people will eventually have heart failure. However, among people aged 70–80 years, the incidence is up to 20–30%. Because the lifespan and generation of multiple risk factors, such as hypertension, diabetes, dyslipidemia, and obesity, are increasing, the survival rate from cardiovascular diseases (such as myocardial infarction, valvular heart disease, and arrhythmia) and the incidence of heart failure are also increasing. In heart failure patients and animal models, autophagosomes accumulate in cardiomyocytes, but the effect of autophagy on heart failure is unclear.

Before heart failure, patients often have cardiac hypertrophy, which is a marker of cardiac remodeling. In a transverse aortic constriction (TAC)-induced mouse myocardial hypertrophy and heart failure model, autophagy markers in myocardiocytes are significantly reduced after TAC but increased with the formation of cardiac hypertrophy in the following weeks. Increased activation of autophagy appears in multiple animal models with heart failure. In cardiomyocytes isolated from rats induced by doxorubicin or diphtheria toxin, many autophagy vacuoles were observed. How is

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autophagy activated during heart failure? In failing cardiac tissue, the damaged mitochondria generate ROS, accompanied by a reduction in ATP levels, promoting AMPK activation. ROS and AMPK both activate autophagy. Moreover, the cAMP/PKA and MAPK/ERK1/2 signaling pathways can activate autophagy. The level of endogenous melanocyte-stimulating hormone in mice induced by TAC and Ang II is associated with the autophagy level. What is the role of autophagy in heart failure? In mice subjected to TAC operation, constriction was remitted 1 week postoperation. The expression of autophagy-related markers was significantly increased, and hypertrophic cardiomyocytes gradually shrunk. When the pressure load is increased, the myocardium shows a compensatory hypertrophy status. Autophagy can increase protein degradation, reduce myocardial hypertrophy, and antagonize ventricular hypertrophy. Double membrane autophagosomes are formed during autophagy for phagocytosis of cytoplasmic proteins and organelles, and then, autophagosomes fuse with lysosomes to form autophagosomes to degrade the contents. The initiation of autophagy is dependent on a series of autophagy-related genes, which form a complex with other proteins. In mammals, Beclin 1 forms a PI3K complex with vacuolar protein sorting protein (VPS) 34 and VPS15, and activation of this complex regulates the formation of autophagosome membranes. Microtubule-associated protein 1 light chain LC3 and ATG12–ATG5–ATG16 are involved in the extension of autophagosome membranes. Changes in the expression of related genes affect autophagy function. Loss of VPS34 in myocytes affects the formation of autophagosomes, leading to mitochondrial network disorders and myocyte contractile dysfunction. Deletion of lysosomal associated membrane protein 2 (LAMP2) affected the fusion between autophagosomes and lysosomes, allowing autophagosomes to accumulate in cardiomyocytes, leading to lethal cardiomyopathy. Global knockout of the lysosomal cysteine endopeptidase cathepsin L resulted in the accumulation of large deformed vesicles in the cytoplasm of cardiomyocytes and led to dilated cardiomyopathy. In cardiomyocytes with a specific deletion of lysosomal deoxyribonucleotide enzyme II, autophagy function was reduced. Heart failure is aggravated under pressure overload. When ATG5 was specifically knocked out in cardiomyocytes, heart failure occurred earlier under pressure load. When autophagy function is reduced, cardiomyocytes cannot scavenge the injured molecules in a timely fashion. For example, mitochondrial DNA binds TLR9 to generate proinflammatory cytokines and promote apoptosis of cardiomyocytes. In addition, increased autophagy of cardiomyocytes under pressure overload can also allow cells to adapt to changes in nutrition and energy requirements. Enhanced autophagy helps maintain ATP levels in cardiomyocytes to maintain the contractility of myocytes. These studies suggest that autophagy in cardiomyocytes plays a key role in preventing heart failure (Lavandero et al. 2015; Shires and Gustafsson 2015).

Autophagy does not always play a protective role during heart failure. There are many forms of cardiomyocyte death in patients with end-stage heart failure. Autophagic cell death promotes the pathological process of heart failure. In patients with heart failure, the loss of cardiomyocytes is mainly attributed to autophagy and swelling, which is closely related to the progress of left ventricular dysfunction

(Kostin et al. 2003). The degree of autophagic activity was correlated with the progression of myocardial hypertrophy and the rate of transformation from myocardial hypertrophy to heart failure in the TAC model of mice. *Beclin*-$1^{+/-}$ mice and myocardial cell-specific Beclin 1 knockout mice have mild contractile dysfunction, and the size of the heart was not affected in the TAC model, while overexpression of Beclin 1 in mouse cardiomyocytes could increase the autophagic activity of myocardial cells under pressure load, which aggravates myocardial hypertrophy and contractile dysfunction. Partial inhibition of autophagy or histone deacetylase inhibitors can alleviate pressure overload-induced heart failure in mice. This suggests that a low level of autophagy is beneficial to the injury of cardiomyocytes under pressure overload, while a high level of autophagy can make the heart more vulnerable to injury and dysfunction. Local but incomplete inhibition of cell autophagy may also be beneficial (Delbridge et al. 2017). Moreover, appropriate autophagy can alleviate tissue injury in heart failure caused by hypertension. However, excessive autophagy promotes macrophage uptake of dead cells, activates inflammation, causes system disorders, and aggravates heart failure. These studies suggest that although autophagy recovers macromolecules and salvages damaged organelles during cell injury, excessive autophagy will degrade proteins and organelles with normal function and inhibit the survival of cardiomyocytes, leading to deterioration of heart failure.

In the course of heart failure, in addition to the autophagy (macroautophagy) mentioned above, mitochondrial autophagy also occurs. Mitochondrial autophagy is a key mitochondrial quality control mechanism in cardiomyocytes, with impaired mitochondrial autophagy leading to abnormal mitochondrial accumulation and subsequent contractile dysfunction. The PTEN-induced protein kinase 1 (PINK1)/ubiquitin ligase Parkin pathway is a key pathway regulating mitochondrial autophagy. PINK1/Parkin is closely related to the occurrence of Parkinson's disease, and studies have shown that PINK1/Parkin-mediated mitochondrial autophagy also affects cardiac function. PINK1 knockout mice exhibited increased mitochondrial dysfunction and oxidative stress, and cardiac hypertrophy occurred at 2 months of age. Although Parkin knockout mice had a normal cardiac function at a young age, abnormal mitochondrial accumulation occurred with age. Loss of the Parkin receptor Mitofusin 2 (MFN2) resulted in decreased Parkin-mediated mitochondrial autophagy in the heart, reduced contractility, increased cardiac hypertrophy, and heart failure (Shires and Gustafsson 2015). A recent study found that the AMPK isoform AMPKα2 is converted to AMPKα1 in the hearts of both patients and TAC mouse models of heart failure, accompanied by a decrease in mitochondrial autophagy and dysregulation of mitochondrial function, mainly due to AMPKα2 specifically binding to PINK1 to promote its phosphorylation of Ser495, thereby increasing mitochondrial autophagy and delaying the onset of chronic heart failure (Wang et al. 2018). In addition to the PINK1/Parkin pathway, receptor-mediated mitochondrial autophagy pathways are also important pathways for the regulation of mitochondrial autophagy. The mitochondrial outer membrane proteins NIX, BNIP3, and the domain on FUNDC1 bind to LC3 and act as receptors for autophagosomes, mediating mitochondrial autophagy. Abnormal mitochondria accumulate in BNIP3 and

NIX-deficient mouse cardiomyocytes, and the mice rapidly develop cardiac dysfunction. In addition, the level of mitochondrial autophagy in cardiac tissue is reduced with age, and injured mitochondria cannot be cleared in time, resulting in excessive ROS production and the oxidative injury of various mitochondrial proteins. Parkin overexpression can reduce age-related heart disease. Although knockout of NIX and BNIP3 caused impaired mitochondrial turnover, mitochondrial autophagy is not all beneficial in the case of stress. Myocardial-specific knockout of NIX attenuated cardiac fibrosis in TAC model mice and inhibited decreased contractile function, whereas cardiomyocyte-specific overexpression of NIX led to the development of cardiomyocyte apoptosis and heart failure. Mitochondrial autophagy is important for maintaining mitochondrial homeostasis. Insufficient autophagy impairs mitochondrial clearance, and then, myocardial ROS accumulation may cause mitochondria-triggered apoptosis. However, excessive mitochondrial autophagy leads to a decrease in mitochondria number, and ATP production is not enough to maintain contraction of cardiomyocytes.

What factors regulate the various aspects of autophagy during heart failure? MicroRNAs (miRNAs) are a class of small noncoding RNAs that are widely distributed in multiple species and tissues and mediate the silencing of target genes in the posttranscriptional or translational phase by interacting with target genes. miRNAs are involved in the regulation of autophagy initiation, vesicle nucleation, vesicle extension, and autophagosome maturation. In cardiovascular disease, multiple autophagy-related genes, such as ATG7, ATG9, LC3, p62, Beclin-1, AMPK, mTOR, etc., can be directly or indirectly regulated by miRNAs. miR-212/132 could directly regulate FoxO3, a transcription factor that promotes hypertrophy and autophagy. miR-212/132 null mice showed increased autophagy in cardiomyocytes in pressure overload-induced heart failure, and the degree of cardiac hypertrophy was significantly reduced, which suggests that myocardial miRNA-212/132 deletion can inhibit cardiac hypertrophy and improve cardiac function by upregulating autophagy (Ucar et al. 2012). miR-221/222 downregulated the expression of LC3II and ATG5 and promoted the expression of SQSTM1/p62 to inhibit autophagy by silencing PTEN and activating the Akt signaling pathway. Myocardial-specific overexpression of miR-221 could reduce autophagic flow and inhibit heart failure by inhibiting the formation of autophagic vesicles. Cardiomyocyte-specific overexpression of miR-222 also inhibited autophagy to promote heart failure. miR-199a regulates the GSK3β/mTOR complex, which in turn regulates autophagy. Myocardial-specific miR-199a transgenic mice showed cardiac hypertrophy with concomitant autophagy. However, overexpression of ATG5 in miR-199a-overexpressing cardiomyocytes reduced the pro-hypertrophic effect of miR-199a (Li et al. 2017). The expression of miR-200b was reduced in the cardiac fibrosis model, which can reduce the autophagy of cardiac fibroblasts by inhibiting the ratio of LC3BII/I and increasing the expression of p62. miR-451 can inhibit the formation of autophagosomes by targeting TSC1, thereby avoiding excessive autophagy under hypertrophic stimulation. Overexpression of miR-451 can inhibit cardiac hypertrophy, while knockout of miR-451 promotes cardiac hypertrophy (Song et al. 2014). miR-30e can inhibit cardiomyocyte

autophagy by downregulating the expression of Beclin 1, thereby mediating the protective effect of angiotensin-converting enzyme 2 (ACE2) in a doxorubicin-induced rat heart failure model (Sun et al. 2018).

It is not clear whether the upregulation of autophagy is meant to repair damaged cardiomyocytes or be an adaptive suicide pathway. Is autophagy a protective mechanism or a pathological injury? Current evidence has indicated that under physiological conditions, autophagy is very important for the stable status of cells, and overactivation of autophagy is harmful (Martinet et al. 2007; Rothermel and Hill 2007). Although the findings on autophagy provide a new therapeutic target, its clinical application requires further development and research. First, the mechanism of autophagy is not completely clear. In addition to mTOR and Beclin-1, other signaling pathways are believed to activate autophagy. Second, the role of autophagy in heart failure is controversial and therefore needs further investigation. The mechanism and regulation of autophagy indicate its role in different stages of heart failure. Selective inhibition or activation of autophagy will be a new treatment to suppress heart failure development.

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Chapter 17
Autophagy, Myocarditis, and Cardiomyopathy

Jie Du, Yan Liu, and Jintao Fu

Abstract Direct damage and immune responses after viral infection lead to myocarditis. Autophagy is involved in both viral clearance and replication. In this chapter, we will briefly describe the role of autophagy in viral myocarditis. In addition, we will discuss the role of autophagy in dilated cardiomyopathy, hypertrophic cardiomyopathy, and diabetic cardiomyopathy.

Keywords Autophagy · Myocarditis · Coxsackievirus · Cardiomyopathy · Diabetes

17.1 Autophagy and Myocarditis

Myocarditis is an inflammatory lesion of the myocardium caused by various factors, which are divided into infectious and noninfectious factors. Infection may be caused by bacteria, viruses, spirochetes, rickettsia, fungi, protozoon, and worms. Noninfectious factors include allergies, allergic reactions (such as rheumatic fever), chemicals, and physical or drug factors (adriamycin). In recent years, myocarditis caused by rheumatic fever and diphtheria has been gradually decreasing, but the incidence of viral myocarditis is significantly increasing. Viral myocarditis is a myocardial local or diffusive, acute or chronic inflammatory lesion caused by viral infection that belongs to infective cardiomyopathy. Approximately 5% of patients have myocarditis in the virus epidemic infection period, and the disease has a dispersed onset. Multiple viral infections can lead to the occurrence of viral myocarditis, especially coxsackievirus B3 (CVB3) infection. The pathogenesis of viral myocarditis is still unclear. The possible pathogenesis includes direct pathogen injury and immune response induced by autologous antigen/antibody. Recently, autophagy has been reported to play a very important role in the pathogenic process of viral myocarditis. Autophagy is part of the host's stress response, such as the unfolded protein response (UPR). Since the clearance of cytoplasmic components is a major function of autophagy, the innate immune

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system activates autophagy to degrade infected viruses. In addition, autophagy promotes antigen processing in the later stages of infection, thereby promoting an adaptive immune response. However, in viral myocarditis, whether autophagy is a cell survival mechanism or the process leading to cell death is inconclusive.

17.1.1 Viral Infection Induces Autophagy

In CVB3-infected HeLa cells, the accumulation of autophagosomes can be observed, which marks the occurrence of autophagy after CVB3 infection.

The results of a study using a mouse model infected with coxsackievirus demonstrated that autophagy is activated in multiple organs. In the heart, liver, and pancreas of mice infected with CVB3, LC3 expression was elevated and autophagosome formation was increased. In addition, in CVB3-infected cardiomyocytes, LC3-II expression was elevated and positively correlated with viral RNA levels. The virus causes autophagy mainly through receptor recognition and endoplasmic reticulum stress. Pattern recognition receptors (PRRs) on cells recognize antigen-related pattern molecules of the virus. For example, Toll-like receptor (TLR) 3 recognizes double-stranded RNA, TLR7, and 8 recognizes single-stranded RNA, and TLR9 recognizes DNA with methylated-CpG sites. TLRs bind to Beclin 1 via a downstream linker molecule, disrupting the interaction of Beclin1 with BCL-2 and ultimately activating autophagy (Choi et al. 2018). CVB3 infection can activate endoplasmic reticulum stress and lead to LC3 accumulation and LC3-I to LC3-II transformation through three signal pathways: PERK/eIF2α, IRE1/XBP1, and ATF6.

17.1.2 The Protective Effect of Autophagy in Viral Myocarditis

Autophagy protects cells from the harmful effects of pathogens in a variety of ways. First, autophagy can deliver the invading virus to the lysosome and directly degrade the virus to achieve viral clearance. Second, autophagy can also present an antigen to MHC-II to activate adaptive immunity. Adaptive immunity is primarily activated in antigen-presenting cells, which are then recognized by T cells. Autophagosomes form new structures in cells such as MHC-II-positive dendritic cells, B cells, and epithelial cells. Blocking autophagy by a PI3K inhibitor or ATG12 knockdown can reduce antigen presentation by MHC-II molecules to CD4+ T cells. TLR3 recognizes viral double-stranded RNA, thereby promoting the release of downstream inflammatory factors and inducing the release of type I interferon against viruses; autophagy dysregulation in CVB3-infected cardiomyocytes inhibits TLR3-mediated antiviral responses, therefore inhibiting viral clearance and increasing myocardial damage during toxemia.

In addition, autophagy can counteract apoptosis and promote cell survival, and decreased autophagy leads to decreased survival of cardiomyocytes after viral infection.

17.1.3 Promoting Effect of Autophagy on Viral Myocarditis

Autophagy also has an unfavorable aspect for viral myocarditis. The virus uses autophagy to escape degradation, promote viral replication and release, and promote the progression of myocarditis.

After virus infection, the cell membrane is reorganized, increasing the number of bilayer membrane vesicles. Thus, the increased formation of autophagosomes is observed. The autophagosomes are continuously extended and envelope the virus to form extracellular microbubbles to escape immune recognition. External microvesicles containing high levels of LC3-II were observed in CVB3-infected cells. RNA viruses can also be replicated using autophagy. The bilayer membrane structure of autophagosomes provides a platform for viral replication and protects against recognition and degradation by the natural immune system. After the cells were infected with CVB3, the autophagosomes increased, and the viral titer also increased. After CVB3 infection in animals, 3-methyladenine (3-MA) was given to block autophagy, and virus synthesis was significantly reduced, confirming that CVB3 replicated by autophagy. In CVB3-infected rat cardiac H9C2 cells, autophagy was activated via calpain, which inhibits caspase3 activation and apoptosis early after infection, thereby providing sufficient time for CVB3 replication (Li et al. 2014). Moreover, CVB3 can replicate in infected cardiomyocytes, its replication ability is higher in cardiac fibroblasts than in cardiomyocytes, and CVB3 infection-mediated autophagosome formation is also observed in cardiac fibroblasts.

On one hand, autophagy can promote the replication of the virus. On the other hand, excessive autophagy can promote the inflammatory reaction. The use of CVB3 to stimulate cardiomyocytes and cardiac fibroblasts can lead to an increase in the production and release of inflammatory factors, thereby aggravating the progression of myocarditis. Inhibition of autophagy can inhibit the production of inflammatory factors. Thus, in the acute viral infection stage, autophagy inhibitors can effectively inhibit viral replication. In the chronic stage, autophagy activators can promote the survival of cardiomyocytes.

17.2 Autophagy and Cardiomyopathy

Cardiomyopathy is a group of progressive cardiac dysfunction lesions caused by structural changes in ventricles and damaged function of the myocardial wall. According to statistics, among admitted patients, cardiomyopathy accounts for 0.6–3.4% of cardiovascular diseases and has recently shown an increasing trend. The

clinical manifestations include enlarged heart, arrhythmia, embolism, and heart failure. Generally, the etiology is associated with viral infection, autoimmune response, heredity, drug poisoning, and abnormal metabolism and can be divided according to pathology into dilated cardiomyopathy, hypertrophic cardiomyopathy, and restrictive cardiomyopathy.

17.2.1 Dilated Cardiomyopathy

The main characteristics of dilated cardiomyopathy are unilateral or bilateral enlarged cardiac chambers and hypofunction in the myocardial systolic phase, which is associated with or without congestive heart failure. The disease is often accompanied by arrhythmia, and its fatality is relatively high. Pathological changes in dilated cardiomyopathy patients indicated that cardiomyocytes show significant vacuolization, atrophy, and degeneration in the cell plasma. Immunohistochemistry has indicated that the levels of lysosome-related membrane proteins and proteases are increased. Degenerated cardiomyocytes show double-layer autophagic vacuoles, suggesting that autophagy participates in the occurrence of dilated cardiomyopathy. Autophagy plays a very important role in maintaining energy metabolism in cardiomyocytes of neonatal mice. Mice with ATG3, ATG5 or ATG7 knockout died immediately after birth. However, excessive activation of autophagy led to cardiomyocyte death in neonatal mice and cardiomyopathy. In the mouse embryonic period, inhibition of autophagy led to cardiomyopathy, and death because of cardiac dilatation after 6 months. In adult mice, autophagy inhibition in cardiomyocytes immediately caused myocardial hypertrophy and dysfunction, accompanied by the accumulation of ubiquitinated proteins. Autophagy participates in scavenging proteins with abnormal structures during cardiomyocyte remodeling. Early aggregation of soluble proteins is very unfavorable. Toxic protein degradation via autophagy or separation of toxic protein bodies can protect cardiomyocytes. Therefore, activation of autophagy in early dilated cardiomyopathy is a protective mechanism by scavenging proteins with an abnormal structure and labeling them by ubiquitin, thus reducing the toxicity of proteins. Desmin cardiomyopathy is a severe myocardial disease caused by a missense mutation of the *CryAB* gene, characterized by the accumulation of misfolded proteins. In this disease, autophagy is an adaptive mechanism that scavenges aggregates of toxic proteins. Hybridization of mice with the *CryAB* missense mutation and mice with *Beclin* $1^{+/-}$ has revealed that a reduction in autophagy aggravates the accumulation of intracellular ubiquitinated proteins, leading to heart failure (Tannous et al. 2008). Myocarditis and dilated cardiomyopathy developed from myocarditis also easily induce heart failure. In heart failure caused by cardiovascular diseases such as dilated cardiomyopathy, moribund cardiomyocytes show significantly enhanced autophagic characteristics. This is because aggregation of misfolded proteins is a strong autophagic inducer, and autophagy adapts to the unfavorable environment by positive feedback and continuous enlargement. Cellular dysfunction by excessive autophagy may be a direct reason for heart failure. This pathological injury

is determined by the degree of autophagy or duration. Excessive autophagy in cardiomyocytes may result in pathological changes. Furthermore, Russo et al. found that myristic acid leads to myocardial hypertrophy by inducing excessive autophagy (Russo et al. 2012). After LC3 is inhibited, autophagy is blocked, and the process of myocardial hypertrophy is moderated, suggesting that overactivation of autophagy could cause myocardial injury.

17.2.2 Hypertrophic Cardiomyopathy

Hypertrophic cardiomyopathy (HCM) is mainly caused by genetic mutations. Although its pathological mechanism is different from that caused by pressure load, the clinical phenotype is similar. Damaged autophagy can be observed in HCM patients carrying an *MYBPC3* gene mutation or in HCM model mice with *Mybpc3* mutation. Autophagic flux is reduced in mice with point mutations, although lysosomal degradation is not influenced. Activation of the Akt-mTORC1 signaling pathway in mutated mice is increased. Rapamycin or energy limitation can inhibit the occurrence of cardiomyopathy in model mice. Rapamycin treatment also overturns HCM caused by specific knockout of the *PTEN* gene. MiR-451 in the heart tissue of pulmonary heart disease is significantly downregulated. MiR-451 can inhibit the expression of TSC1, which positively regulates autophagy. This is one of the mechanisms for increased autophagy in the HCM heart.

17.2.3 Diabetic Cardiomyopathy

Diabetes is a metabolic disease caused by insufficient insulin secretion or reduced sensitivity, which is manifested by an increase in plasma glucose levels. Diabetes can cause multiple complications, and nearly 80% of diabetes-related deaths are caused by cardiovascular complications. Diabetic cardiomyopathy is a myocardial disease occurring in diabetes that cannot be interpreted by hypertension, coronary heart disease, valvular heart disease, and other heart diseases. Hyperglycemia and hyperinsulinism lead to metabolic changes in cardiomyocytes and further promote extensive focal myocardial necrosis, showing subclinical cardiac dysfunction and finally developing into heart failure, arrhythmia, cardiogenic shock, or even sudden death when severe. The known pathogenesis of diabetic cardiomyopathy may be related to cardiomyocyte metabolic disorder, cardiomyocyte calcium transport defect, microangiopathy in the coronary artery, myocardial interstitial fibrosis, and cardiac autonomic neuropathy. A recent study has indicated that autophagy may be a new mechanism participating in the onset of cardiomyopathy in diabetes patients. In the type 1 diabetes model, cardiac dysfunction was related to myocardial autophagy inhibition. Metformin treatment of OVE26 and STZ diabetic mice reduced myocardial injury. Similarly, overexpression of heme oxygenase-1 (HO-1) or mitochondrial

aldehyde dehydrogenase in STZ diabetic mice protected the myocardium. Activation of AMPK and autophagy may be the mechanism. Under high glucose, AMPK activity was inhibited, leading to autophagy disorder. Metformin, HO-1 or aldehyde dehydrogenase enhanced the autophagy capability and improved cardiac function by recovering AMPK activity, suggesting that autophagy is conducive to the heart of most type 1 diabetes patients.

How does autophagy participate in the pathological process of diabetic cardiomyopathy? In diabetic patients, a long-term increase in blood glucose leads to a decrease in the number of glucose transporters and their receptors in the cardiomyocytes, which affects the transport of glucose into the myocardium, resulting in insufficient myocardial energy. Starvation induces autophagy and clearance of abnormal organelles and cytoplasmic proteins. If autophagy is inhibited, such as ATG7 deletion, high levels of polymerized protein appear in the cells, affecting cell metabolism, and promoting apoptosis. However, autophagy is involved in oxidative stress damage in diabetic cardiomyocytes. Hyperglycemia promotes the production of ROS and inhibits the clearance of ROS, leading to the accumulation of ROS in cardiomyocytes, and oxidative stress-induced cardiomyocyte injury is an important pathogenic factor of diabetic cardiomyopathy. Nuclear factor E2-related factor (Nrf2) is a key factor in the cellular oxidative stress response and regulates the expression of antioxidant enzymes and phase II detoxification enzymes by interacting with antioxidant response elements (ARE). Nrf2 protein expression is significantly reduced in the heart of diabetic patients. As a bridge between autophagy-associated proteins, LC3 and polyubiquitinated proteins, p62 transports aggregated proteins, damaged mitochondria, etc. to autophagosomes by ubiquitination, which will reduce ROS damage to cardiomyocytes. P62 also activates the Nrf2 signaling pathway through Keap1, thereby inhibiting oxidative stress. Thus, autophagic activation can protect cardiomyocytes under diabetic conditions by inhibiting oxidative stress.

However, multiple studies have indicated that autophagy has poor effects on the heart of type 2 diabetes patients. Feeding mice a diet containing 60% fructose for 12 weeks to induce type 2 diabetes caused myocardial insulin resistance. The onset of resistance positively correlates with the aggregation of autophagosomes. Insulin-induced activation of the PI3K/AKT signaling pathway had a negative regulatory effect on autophagy by mTOR. The PI3K/AKT signaling pathway in the myocardium of a type 2 diabetes mouse model was downregulated, which further activated autophagy and aggravated myocardial insulin resistance. Thus, while suffering from type 1 or 2 diabetes, cardiac autophagy and the regulatory mechanisms may be different. The former activates mTOR and weakens autologous phagocytosis by inhibiting AMPK. The latter inhibits mTOR and enhances autologous phagocytosis by inhibiting PI3K/AKT. Because the conclusions are controversial, more studies are required to elucidate the effect and mechanism of autophagy in different diabetes models.

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Chapter 18
Autophagy, Hyperlipidemia, and Atherosclerosis

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Abstract Autophagy is an evolutionarily conserved process in eukaryotes that processes the turnover of intracellular substances. Atherosclerosis is a disease caused by multiple factors, it mainly occurs on the walls of large and medium blood vessels and atherosclerotic plaques form in the intima of the blood vessels. Hyperlipidemia is considered to be a very dangerous factor leading to cardiovascular and cerebrovascular diseases, especially atherosclerosis. This chapter mainly introduces the key role of autophagy in hyperlipidemia and atherosclerosis, that is, impaired lipophagy affects the degradation of triacylglycerol, cholesterol, etc., leading to hyperlipidemia in atherosclerosis. In patients, excessive levels of autophagy accelerate the rupture of atherosclerotic plaque. This chapter also describes the advances in the treatment of atherosclerosis and hyperlipidemia by targeted autophagy.

Keywords Autophagy · Hyperlipidemia · Atherosclerosis · Vascular endothelial cells

Hyperlipidemia is relatively common in the general population and is a common disease in middle-aged and elderly people. It is also a disease that has received attention, as it seriously affects the normal life of many middle-aged and elderly people. Hyperlipidemia is considered to be a significant risk factor for cardiovascular and cerebrovascular diseases, especially atherosclerosis. Excessive blood lipids can cause disorders in lipid metabolism, increased blood viscosity, and lipid deposits in the intima of blood vessel walls, which gradually accumulate into atherosclerotic plaques. Plaques continually increase, gradually occluding blood vessels, resulting in narrowing of the blood vessel lumen and poor blood circulation, which can lead to serious consequences, particularly if the blood supply to vital organs becomes insufficient. Severe, cardiovascular atherosclerosis can cause coronary heart disease, myocardial infarction, angina pectoris, cerebral thrombosis, cerebral hemorrhage,

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and stroke, which can prove life-threatening. Under environmental stimuli such as energy deficiency, hypoxia, oxidative stress, and other metabolic pressures, cells can degrade proteins and organelles in lysosomes by autophagy and thereby obtain the nutrients such as amino acids, fatty acids, and nucleic acids that are necessary to resist negative effects of an unhealthy environment and promote cell survival. Autophagy is mainly involved in maintaining the steady state of normal cells. When stimulated, autophagy can self-reassort to drive cell survival. Current research has demonstrated that autophagy plays an important role in cardiovascular diseases such as hyperlipidemia and atherosclerosis.

18.1 Autophagy and Hyperlipidemia

18.1.1 Hyperlipidemia

Hyperlipidemia is the most common form of dyslipidemia. Hyperlipidemia is divided into two subtypes: primary and secondary. Primary hyperlipidemia, also known as familial hyperlipidemia, is due to single or multiple defects in the genes involved in lipoprotein transport. In addition, metabolic abnormalities caused by abnormal receptors, enzymes, or apolipoproteins or due to environmental factors (diet, nutrition, or drugs) and mediated through unknown mechanisms are attributed to the primary disease. Secondary hyperlipidemia, which is also called acquired hyperlipidemia, is caused by changes in blood lipids and lipid metabolism often due to other dysfunctions (such as diabetes, high blood pressure, and obesity).

Hyperlipidemia can also be classified according to the type of lipids elevated in the blood and includes conditions such as hypercholesterolemia, hypertriglyceridemia, high cholesterol, and high triglyceride mixed hyperlipidemia. Elevated low-density lipoprotein (LDL) cholesterol is also classified as a type of hyperlipidemia.

Hyperlipidemia is relatively common in the general population and is considered to be a significant risk factor for cardiovascular and cerebrovascular diseases. In addition, many forms of hyperlipidemia can lead to diseases such as fatty liver, cirrhosis, hypertension, diabetes, hyperuricemia, and acute pancreatitis.

18.1.2 Lipid Droplets

Lipid droplets (LDs) are present in all types of mammalian cells. They are composed of a lipid core (triglycerides and cholesterol) and a surrounding monolayer of phospholipids, which separates the core from the hydrophilic intracellular environment. In addition, the droplet surface is coated with structural proteins, called lipid-coated proteins. LDs function as reservoirs for storing excess lipids in the body, and, in recent years, it has been found that there are many specific LD-related proteins

in cells. Therefore, LDs are considered independent organelles with multiple cell functions.

In the early stage of LDs formation, various organelles such as the endoplasmic reticulum, mitochondria, and peroxisomes surround LDs, and they all play an important role in the formation of LDs. Through the action of various enzymes present on these organelles, lipids are processed and packaged by a series of biochemical processes, such as esterification, and finally wrapped and presented to primary LDs for storage. The lipids encapsulated in the LDs gradually increase and mature, and the composition of the related proteins also changes accordingly. The ability of LDs to decompose is closely related to their size. The larger the LDs are, the more difficulty they have undergoing decomposition. This regulated feature gives the organism a survival advantage during starvation. The smaller droplets have a relatively large surface area onto which lipases can easily bind to degrade triglycerides and cholesterol. Lysosomal membranes can be recycled during autophagy and thus participate in the production of new lysosomes, a process known as autophagic lysosomal regeneration. Under the action of dynamin 2 (DNM2), small vesicles form a tubular structure by budding and detaching from autophagosomes, forming a prelysosomal structure that maintains efficient autophagy in liver cells under nutritional constraints throughout the maturation process.

18.1.3 Lipid Autophagy

Lipophagy refers to the process by which cells degrade triglycerides and cholesterol stored in LDs through selective lysosome-dependent macroautophagy. The autophagosome of the bilayer membrane envelops a portion of a large LDs or an entire small LDs, wherein the lipids can be entrapped by the autophagosome through other cytoplasmic components. Subsequently, the autophagosome encapsulating the LDs is fused with a lysosome to decompose the lipid droplets by hydrolysis with lysosomal acid hydrolase. After the LDs are decomposed, their degradation products are released into the cytoplasm and include free fatty acids (FFAs), which maintain mitochondrial β-oxidation to produce the ATP that maintains energy balance within the cell (Liu and Czaja 2013a). Lipid autophagy directly affects the intracellular energy balance by degrading lipids, which indirectly regulates the organism's food intake needs. Impaired lipid autophagy has been found to cause many important metabolic disorders, such as fatty liver, obesity, and atherosclerosis.

18.1.4 The Selectivity of Lipid Autophagy

Lipid autophagy is selective macroautophagy, and the autophagic degradation of LDs is recognized and initiated by specific proteins. However, it is not clear whether the LDs are recognized as autophagic substrates or whether a certain number of LDs

are regulated by signaling invoked by the cellular nutrient state to initiate targeted degradation. Lipid autophagy delivers LDs to the lysis compartment for degradation and is mediated by the action of autophagy-related proteins and LD-related proteins. Possible candidates for mediating LD targeting are soluble NSF attachment protein receptors (SNAREs), and current studies have found that SNAREs may play a role in LDs recognition. SNAREs mediate not only fusion between LDs in the cytoplasm but also the production of autophagosomes, as shown in recent studies (Liu and Czaja 2013a). Another possible factor is microtubule-associated protein 1 light chain 3 (LC3). Studies have shown that in the absence of autophagic small body membranes, LC3 interacts with LDs, suggesting that LC3 may play an important role in LDs identification (Liu and Czaja 2013a). Another study showed that inhibition of autophagy can inhibit the formation of LDs. LC3 can colocalize with autophagosomes. In the liver and heart tissues of Atg7-deficient mice, LC3 and LDs were colocalized under starvation conditions. Recently, some important evidence has suggested that the LD-related Ras-associated protein 7 (Rab7), the LD-coated protein perilipin A, TIP47 (tail-interacting protein), and the lipid drop-coating protein perilipin 2 (ADRP) may enter the membrane-bound organelle. Lipid degradation is responsible for the autophagic internalization of vacuoles (Zhang et al. 2018b).

18.1.5 The Function of Lipid Autophagy

Current research has revealed that lipid autophagy has different functions in cell physiology and pathology:

(a) Lipid autophagy is involved in the regulation of intracellular lipid content and energy balance. Impaired lipid autophagy can lead to excessive lipid accumulation in the cell, and defects in autophagy of fat in the liver can lead to fatty liver.
(b) Decomposition products from lipid autophagy (such as FFAs) affect physiological cell activity (cell survival versus death). The most important role for stimulated resistance and cell transdifferentiation is that of maintaining high levels of β-oxidation to provide energy.
(c) Other functions remain largely undefined, such as providing lipids that serve as cellular structural components and regulating lipid-dependent cell signaling (Liu and Czaja 2013a).

Although lipid autophagy occurs in all types of cells, current studies have shown that lipid autophagy plays different roles in different tissues and cells. In hypothalamic neurons, for example, lipid autophagy plays a role in regulating systemic energy storage. The hypothalamus is an important regulator of systemic energy balance, and stored lipids combine with various nutrient signals to determine food intake required by the organism. Arcuate neurons of the hypothalamus regulate feeding through two groups of neurons with opposite functions: anabolic agouti-related peptide (AgRP) neurons express AgRP and neuropeptide Y to stimulate feeding and reduce energy

expenditure, and catabolic proopiomelanocortin (POMC) neurons express POMC, which is processed into the α-melanocyte-stimulating hormone that inhibits eating. When the organism is in an acute starvation condition, FFAs stored in fat cells are released into serum, resulting in an increase in FFA levels in AgRP neurons, and the elevated FFAs are encapsulated in the LDs. Starvation-induced autophagy is able to break down LDs to produce FFAs, which further stimulate the production of AgRP, which increases feeding by directly or indirectly inhibiting POMC neurons. Elevated levels of autophagy in hypothalamic AgRP neurons eventually translate into increased food intake and body weight. In mice fed long term with a high-fat diet, the level of autophagy in the hypothalamus was significantly reduced. Reduced levels of autophagy induce an inflammatory response that activates the IκB kinase β (IKKb)/NF-κB signaling pathway, which in turn increases food intake and body weight. In this respect, autophagic regulation in cell and body energy balance not only limits the degradation of macromolecules or stored cellular materials to obtain energy but it also has a more comprehensive regulatory function that includes modulating food intake (Liu and Czaja 2013a).

In macrophages, lipids can be effluxed by lysosomal acid lipases that degrade LDs through the action of ATP binding cassette transporter A1 (ABCA1). When macrophages initiate a large increase in atherosclerotic lipoproteins, autophagy-dependent cholesterol efflux is enhanced. The LDs are presented to the lysosome through autophagy, and then cholesterol esters (CEs) are degraded by the acid lipase in the lysosome such that the free cholesterol that is generated leaves the lysosome in an ABCA1-dependent manner. This process is specifically activated when the cholesterol level in macrophages is increased. Increasing cholesterol efflux is a potential way to inhibit macrophage formation of foam cells and to reduce production of atherosclerotic lesions. However, activating macrophage autophagy to promote cholesterol efflux through drug treatment is not a viable means of inhibiting atherosclerosis because autophagy also triggers the macrophage inflammatory response, which in turn deleteriously affects arteries. The formation of atherosclerotic plaques, while promoting the breakdown of autophagic cholesterol lipids, increases the level of free cholesterol, which also adversely affects cells.

18.1.6 The Role of Autophagy in Lipid Metabolism

At physiological levels, basal levels of autophagy are involved in lipid metabolism processes, and when lipid levels are increased or starvation conditions are induced in a short period of time, the level of autophagy is increased to decompose LDs, produce fatty acids, and stimulate β-oxidation, among other functions. Inhibition of autophagy can hinder the degradation of lipids and increase intracellular lipid content. As the lipid content in the cytoplasm continues to increase (such as during long-term high-fat feeding), autophagy is inhibited. The process, in turn, exacerbates lipid accumulation and increases the volume of LDs. The liver plays an important role in regulating systemic cholesterol homeostasis. Hepatocytes maintain the homeostasis

of cellular cholesterol by coordinating the control of several cholesterol input and elimination pathways. Hepatocytes acquire cholesterol primarily through de novo synthesis and receptor-mediated lipoprotein uptake from circulation. These input pathways are primarily regulated by the sterol regulatory element-binding protein-2 (SREBP-2)-mediated cholesterol sensing mechanism. SREBP-2 is synthesized as a precursor protein that is retained in the endoplasmic reticulum (ER) membrane and is associated with the sterol-induced SREBP cleavage-activating protein (SCAP). When cellular cholesterol levels are high, cholesterol binds to SCAP, resulting in a conformational change that induces the interaction of SCAP with the ER membrane insulin-inducible gene protein (Insigs), which retains the SREBP-2-SCAP complex in the ER. The reduction in cellular cholesterol promotes translocation of the SREBP-2-SCAP complex to the Golgi where SREBP-2 is proteolytically cleaved. The release of truncated and mature SREBP-2 into the nucleus induces a number of cholesterol synthesis and transport genes, including those encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) and low-density lipoprotein receptor (LDLR). Activation of these genes increases intracellular cholesterol levels, which in turn inhibits SREBP-2 cleavage via a negative feedback loop (Wang et al. 2018b).

18.1.7 Double Regulation of Lipids During Autophagy

Although autophagy mediates lipid metabolism, differences in intracellular lipid content also affect levels of autophagy. Lipids play a pleiotropic role in the control of autophagy, a process involving remodeling of the inner membrane of the cell. The core of this regulation is based at the membrane–cytosol interface where multiple lipids (mainly phospholipids) are directly involved; for example, protein scaffolds are assembled to regulate various steps of the autophagy process. Lipids and lipid metabolic enzymes mediate autophagy processes by controlling at least four fundamental aspects. First, they regulate the cascade of signals that converge on the mTOR pathway, such as phosphatidylinositol 3-kinase (PI3K), which in turn negatively regulates the initiation of autophagy; second, lipids serve as a local signal during membrane binding and thus controls membrane kinetics specifically by mediating membrane deformation and expansion through specific molecule recruitment. A typical example of such regulation is illustrated by the action of phosphatidylinositol-3-phosphate (PI3P). In addition, covalent attachment of PE-containing phospholipids to members of the Atg8/LC3 family stably anchors these key factors to the phagocytic membrane where they regulate protein extension and ultimately block their activation. Finally, lipids can control membrane kinetics by directly affecting the physicochemical properties of the lipid bilayer independent of a protein effector. Many studies have shown that dietary intake of lipids promotes autophagy. Studies in a variety of tissues and cells (neural cells, muscle tissue, pancreas, mammary epithelial cells, liver-derived cells, and colon cancer cells) have shown that elevated levels of FFAs can promote autophagy (Tang et al. 2011). At present, there are relatively few studies on the molecular mechanism of lipid activation in autophagy.

It is known that in pancreatic β cells, lipids activate the c-Jun N-terminal kinase 1 pathway in the endoplasmic reticulum in an oxidative stress-independent manner to induce autophagy.

However, an almost equal numbers of studies have shown that high doses or special types of lipid treatment can inhibit levels of autophagy. Unsaturated FFAs such as oleic acid can increase the level of autophagy of various cells within a certain concentration range (Singh et al. 2009); in contrast, saturated FFAs such as palmitic acid is able to inhibit the level of autophagy in cells, probably because they are less encapsulated in LDs and have a higher concentration in the cytoplasm. In obese mice, the level of autophagy in the liver is relatively low (Yang et al. 2010). In the animal model of long-term high-fat feeding, the level of autophagy increased significantly in the first week, but then the level of autophagy gradually decreased. This reduction in the level of autophagy causes a progressive increase in LDs volume, which ultimately leads to hepatotoxicity and steatosis (Koga et al. 2010; Mei et al. 2011).

Under conditions of obesity or high-fat feeding, the reduction in levels of autophagy may be due to three mechanisms: (a) obesity or high-fat feeding may downregulate the expression of multiple autophagy-related genes, resulting in reduced autophagosome formation and autophagy (Yang et al. 2010); (b) the liver may form more autophagosomes and autophagic lysosomes, but functional defects in lysosomes cause a decrease levels of autophagy and inhibit autophagic degradation (Inami et al. 2011); and (c) impaired fusion of autophagosomes with lysosomes may be regulated by elevated FFAs (Koga et al. 2010).

Lipid metabolism in the liver is mainly divided into the following types: (a) Background-level lipid autophagy affects fat droplets in all tissues, including liver tissue. (b) Induction of lipid-stimulating conditions such as autophagy, long-term starvation or persistent lipid stress activates liver lipid autophagy, thereby regulating the growth of LD. Damage that prevents stimulation of autophagy leads to fatty liver. (c) To maintain a balance between lipid formation and hydrolysis, autophagy may be involved in the formation of LD by some currently unknown mechanism, and partial inhibition of autophagy disrupts the inherent balance between lipid regeneration and degradation.

18.1.8 The Relationship Between Autophagy and Hyperlipidemia

Studies have shown that early hypercholesterolemia in pigs is often accompanied by overactivated mammalian/mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) or complex 2 (mTORC2) signaling pathways. The energy and growth factor response kinase mTOR controls cellular responses changing nutritional status by regulating intracellular ATP, glucose, and amino acid levels. The authors' study showed that compared with normal cholesterol-level controls, after 4 weeks of high-fat/high-cholesterol feeding, Yucatan pigs experienced hypercholesterolemia in the

nonischemic left ventricle. The total mTOR and Raptor levels had risen sharply, which is consistent with the cholesterol levels found among pigs of the Yucatan Peninsula. In addition, in the cardiomyocytes of the hypercholesterolemia group, a large amount of mTOR had accumulated around nuclei. Hypercholesterolemia is also accompanied by overactivation of the upstream and downstream signaling molecules of mTORC1 and mTORC2 complexes, including myocardial Akt, S6K1, 4EBP1, S6, and PKC-α, which increase the expression level of cardiac hypertrophic markers and alter myocardial metabolism. The effects of hypercholesterolemia on these heart-related signals make the myocardium particularly susceptible to ischemic injury and reduce the level of autophagy in cardiomyocytes (Glazer et al. 2009).

Subsequent studies have shown that inhibition of mTORC1 activity in mouse liver enhances the ability of mice to resist hepatic lipidosis and hypercholesterolemia caused by high-fat/high-cholesterol feeding. In the liver of mice with conditionally knocked out Raptor, the mTORC1/PI3K/AKT signaling pathway in the liver was greatly disrupted. After high-fat/high-cholesterol feeding, the conditional knockout Raptor mice had not only a significant decrease in body weight but also a significant decrease in triglyceride and plasma cholesterol levels in the liver compared with the wild-type (WT) mice. Moreover, this reduction depended on lipid 1 (lipin1). When the expression of lipin1 was specifically knocked down by shRNA in mouse liver cells with conditionally knocked out Raptor, the decrease in triglyceride and cholesterol levels induced by the conditional knockout of Raptor was offset.

In hyperlipidemic mice, macrophages that specifically lack caspase-1/11 are able to reduce cholesterol crystallization and hepatitis. This reduction is accompanied by an increase in the level of autophagy (Hendrikx et al. 2013). The authors transplanted bone marrow from WT mice or caspase-1/11-/- (dKO) mice into Ldlr-/- mice and then fed a high-fat/high-cholesterol diet for 12 weeks. The liver in the small dKO mice showed a significantly weaker inflammatory response. Compared with the isolated Kupffer cells (KCs) of WT mice, mouse-derived dKO KCs showed decreased cholesterol crystallization, increased cholesterol efflux, and elevated levels of autophagy. In vitro studies have shown that, in bone marrow-derived macrophages (BMDMs) of WT mice, oxidized low-density lipoprotein (ox-LDL) treatment significantly impaired autophagic action, while BMDMs originating from dKO mice had a normal level of autophagy.

In addition, in human retinal capillary pericytes (HRCPs), highly oxidized glycated LDL (HOG-LDL) treatment activated both oxidative and endoplasmic reticulum stress. This stress eventually leads to mitochondrial dysfunction, apoptosis, and autophagy. HOG-LDL treatment significantly increased the protein expression levels of LC3-II, beclin-1, and ATG5 in HRCP. In addition, when oxidative stress, endoplasmic reticulum stress, and mitochondrial dysfunction were inhibited, the autophagy induced by HOG-LDL was also significantly attenuated. This finding suggests that autophagy is induced by HOG-LDL at least partially downstream of the initial responses to oxidative stress, endoplasmic reticulum stress, and mitochondrial dysfunction (Fu et al. 2012). When HRCPs are stimulated by HOG-LDL, autophagy may play a dual role by promoting cell survival under mild and moderate stress but leading to cell death when pressure is increased.

In adult Sprague Dawley rats, when the anti-lipolytic agent 3,5-dimethylpyrazole (DMP) was used to promote macroautophagy, age-induced high-cholesterol blood levels in rats were reduced. Aging humans and rodents often experience a decline in metabolic function and the development of many age-related diseases, including hypercholesterolemia, which is a major risk factor for atherosclerosis and cardiovascular disease. Age-related, total, and LDL cholesterol levels were also increased, which is in accordance with the incidence of cardiovascular disease. In addition, aging is often accompanied by a decrease in the level of autophagy. When adult Sprague Dawley rats were fed overnight and injected with a certain amount of DMP to induce acute macroautophagy, the levels of total LDL and high-density lipoprotein (HDLs) cholesterol in the blood were reduced to the levels typical of young rats, and triglyceride levels even dropped below the level of young rats. DMP stimulation was able to improve the age-dependent plasma lipid changes in rats over a short period of time. The cholesterol-lowering effect of DMP was independent of the activation status of age-related 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA-R). It was also associated with an increase in age-related lipid peroxidation and was involved in the recovery of only a number of LDL ginsenosides, the main active component of ginseng, which can play a role in anti-inflammatory, antitumor, and NAFLD prevention. Rb2 is considered to be the most abundant ginsenoside in ginseng, which ameliorated hyperlipidemia in streptozotocin-diabetic rats. Rb2 can attenuate lipid accumulation in the liver by inducing autophagy through induction of silent information regulator 1 (sirt1) and activating AMPK, which leads to an improvement in NAFLD and glucose tolerance. Resveratrol (RSV) is a natural polyphenol that has been found to be beneficial for the treatment of many metabolic diseases. Studies have shown that RSV may reduce cells by inducing autophagy in hepatocytes through activation of the cAMP-PKA-AMPK-SIRT1 signaling pathway. The internal lipid content stimulates fatty acid β-oxidation and affects hyperlipidemia.

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is a multifunctional serine/threonine kinase involved in a variety of cardiovascular diseases. Increased inflammatory response and activation of MAPK and NF-κB signaling pathways, excessive oxidative stress, ER stress, and autophagy were also observed in palmitate-treated H9C2 cells, whereas pretreatment with CaMKII inhibitors reduced these pathological signals. TLR4 is an upstream signal for CaMKII in palmitate-treated H9C2 cells. Mass spectrometry of serum lipids (FFAs, TGs, and TCs) and blood glucose levels were significantly increased in apolipoprotein E-deficient (ApoE-/-) mice fed a high-fat diet compared to mice fed a normal diet. Therefore, CaMKII plays a key role in the pathogenesis of obesity/hyperlipidemia-induced cardiac remodeling (Zhong et al. 2017b).

Recent studies have shown that the antioxidant catalase can remedy cardiac dysfunction caused by high-fat feeding by regulating autophagy in an IKKβ-AMPK-dependent manner. WT mice can have obesity, hyperinsulinemia, and hypertriglyceridemia after 20 weeks on a high-fat diet. However, the above symptoms did not occur in transgenic mice overexpressing catalase in the heart. High-fat diet feeding also caused cardiac hypertrophy, increased left ventricular end-systolic and diastolic diameter, promoted reactive oxygen species (ROS) production, and inhibited

autophagy in myocardial cells in WT mice. Transgenic catalase can significantly attenuate the above symptoms. Additional mechanism studies in vivo have shown that catalase can attenuate the phosphorylation of inhibitor kappa B kinase β (IKKβ), AMP-activated protein kinase (AMPK), and tuberous sclerosis 2 (TSC2) induced by high-fat feeding and ultimately inhibit activation of mTOR that is induced by high-fat feeding. Studies have shown that palmitic acid can reduce the level of autophagy and contractile function of cardiomyocytes in vitro, a finding that is consistent with the results of high-fat feeding in vivo. When inhibiting IKKβ, activating AMPK or inducing autophagy, the effect of palmitic acid is significantly reduced (Liang et al. 2015).

Increasing the intake of branched-chain amino acids (BCAAs) reduces body weight, whereas elevated circulating BCAAs are associated with NAFLD. Recent studies have shown that, in fat cells, BCAA activates AMPKα2 and stimulates lipolysis, thereby increasing plasma FFAs, which in turn leads to accumulation of FFAs in the liver. In the liver, BCAA activates mTOR, which inhibits FFA-to-TG transformation and autophagy. Inhibition of FFA-to-TG conversion blocks the liver FFA efflux pathway and enhances FFA lipid toxicity. Blocking autophagy impedes self-repair mechanisms, prevents lipotoxicity, and increases apoptotic cell death (Zhang et al. 2016).

Autophagy-related gene 14 (Atg14) plays an important role in autophagy and lipid metabolism in the liver, and Atg14 functions are regulated by the forkhead box O (FoxO) transcription factors and biological cycles. Regulators of circadian rhythms, FoxOs, and biological cycle rhythms affect the expression of Atg14, which in turn promotes autophagy. Atg14 is an essential gene in the initiation of autophagy. Recent studies have shown that knocking out Atg14 in primary liver cells significantly reduces levels of autophagy in mouse liver cells. Further studies have shown that conditional knockout of Atg14 in mouse liver increases levels of triglycerides in the liver and serum. Conversely, overexpression of Atg14 in liver cells significantly increased levels of autophagy, and overexpression of Atg14 in the liver significantly increased liver levels of autophagy in WT and FoxO1/3/4 conditional knockout mice and ameliorated hypertriglyceridemia caused by high-fat feeding.

Endothelial progenitor cells (EPCs) play a major role in maintaining endothelial integrity and contribute to the regeneration of damaged blood vessels and the reconstruction of ischemic tissues. Some researchers have used ox-LDL to mimic hypercholesterolemia in rat bone marrow-derived EPCs. ox-LDL activates autophagic flux in a dose-dependent manner while inhibiting EPC proliferation. The inhibition of autophagy by silencing Atg7 or by treatment with 3-methyladenine further exacerbated the inhibited proliferation of ox-LDL, indicating a protective effect of autophagy on ox-LDL. This finding provides new insights into the important role of autophagy in maintaining proliferation and promoting the viability of EPCs and may be beneficial for improving EPC transplantation efficacy and enhancing revascularization of patients with hypercholesterolemia (Yang et al. 2017).

Irbesartan (Irb) acts as a selective peroxisome proliferator-activated receptor (PPAR-γ) agonist with anti-inflammatory and antioxidative functions, which it uses

in glucose and lipid metabolism. Studies have shown that Irb can improve hyperlipidemia and hepatic steatosis by upregulating the expression of PPAR-γ, thereby activating the AMPK/Akt/mTOR signaling pathway and inducing autophagy in the liver (Zhong et al. 2017a).

Coenzyme Q10 (CoQ10) is a fat-soluble anthraquinone compound found in nature. Also known as "ubiquinone," it is a key intermediate in the synthesis of adenosine triphosphate (ATP) through mitochondrial calcium-dependent ion channels. Coenzyme Q10 can reduce the expression of tumor necrosis factor (TNF)-α and interleukin (IL)-6 genes in heart tissue of ApoE-/- mice, reduce the number of phagocytes in heart tissue of ApoE-/- mice fed with HD, and reduce p62-induced and increase LC3-induced expression and phosphorylation of ERK in cardiac tissue. Therefore, CoQ10 helps to alleviate hyperlipidemic heart damage, such as LDs downregulation, pro-inflammatory gene expression, macrophage accumulation, and upregulation of autophagy (Zhang et al. 2018a).

Type 2 diabetes mellitus (T2DM) is an epidemic disease worldwide in which insulin resistance is initially developed with significant islet β-islet overexpansion and hyperinsulinemia. As the disease progresses, pancreatic β cells are overwhelmed and cannot compensate for the insulin resistance. In addition, T2DM is often associated with other metabolic diseases, such as hyperlipidemia, obesity, and metabolic syndrome. During progression of T2DM, the mTORC1 signaling pathway is chronically activated, which induces senescence and acts as an endogenous inhibitor of autophagy. Some researchers (Guillen and Benito 2018) have observed mTORC1 overactivation in some tissues affected by T2DM; for example, it leads to dysfunction in the myocardium. In the liver, in addition to participating in the regulation of liposome homeostasis, mTORC1 overactivation can promote lipogenesis and inhibit lipolysis and fat deposition, and insulin resistance is upregulated, leading to glucose imbalance and liposome homeostasis. In adipose tissue, mTORC1 is also involved in insulin resistance.

Fenofibrate is a fibric acid derivative that acts as a peroxisome proliferator-activated receptor-α agonist. As a PPAR-α agonist, fenofibrate was used to treat hyperlipidemia and hypercholesterolemia in the 1970s. In recent years, it has been found to have anticancer functions, inhibiting the growth of cancer cells, the movement and metastasis of cancer cells, and tumor angiogenesis. In a recent study, fenofibrate was also found to induce autophagy in normal cells. In retinal pigment epithelial cells, fenofibrate induces autophagy to relieve cellular damage caused by hyperglycemia and hypoxia. In cardiomyocytes of diabetic mice, fenofibrate induces autophagy to prevent fibrosis and inflammation. However, after chronic activation of PPARα with fenofibrate, mouse liver protein levels associated with autophagy were significantly reduced, and autophagy was eventually inhibited. These conflicting results indicate that fenofibrate may be dependent on autophagy in the cell. Recent studies have shown (Tao et al. 2018) that fenofibrate induces autophagy by modulating the AMPK-mTOR pathway, ultimately blocking complete autophagic flux in PCa cells; fenofibrate treatment produces self-regulating metabolites that induce endoplasmic reticulum stress in prostate cancer cells and promote apoptosis.

Recent studies have shown that the direct interaction of proprotein convertase subtilisin/kexin type 9 (PCSK9) with apolipoprotein B (apoB) can inhibit the autophagic degradation of apoB. Increased lipoprotein secretion caused by apoB ultimately leads to increases in blood cholesterol and triglyceride levels (Sun et al. 2012). Among drug therapies, statins remain first-line treatments, but some clinical studies have shown that some patients with high risk of cardiovascular disease have reduced lipid levels after receiving adequate statin therapy. Although not obvious, there is still a high risk of cardiovascular disease. In addition, some patients are intolerant of high-dose statin treatments. PCSK-9 inhibitors can be used in this population (Chaudhary et al. 2017) to address the therapeutic deficiencies of statins.

18.1.9 Conclusion

In summary, current research indicates a very close and complex relationship between autophagy and hyperlipidemia. The difference in intracellular lipid content affects the level of autophagy in cells. Short-term lipid stimulation can significantly promote autophagy, but long-term or specific types of lipid stimulation can inhibit autophagy levels, and studies have shown that aging and hyperlipidemia caused by high-fat feeding is often accompanied by lower levels of autophagy in vivo. Autophagy regulates lipid metabolism by means of lipid autophagy, which in turn affects the content of cholesterol and triglycerides in cells. Inducing autophagy can significantly reduce the lipid content in cells and in mice. Key factors such as Atg14, mTOR, and LC3 may play an important role in the interaction between autophagy and hyperlipidemia. However, the specific molecular mechanism associated with autophagy and hyperlipidemia remains to be discovered. Studies have shown that the promotion of macroautophagy can significantly reduce blood lipid levels in vivo, suggesting that targeted promotion of autophagy may be an effective way to treat hyperlipidemia, providing a new choice for the treatment of hyperlipidemia.

18.2 Autophagy and Atherosclerosis

Atherosclerosis occurs mainly on the walls of large and medium blood vessels. Atherosclerotic plaques form in the intima of blood vessels. The formation and development of plaques is a very complex process involving multiple cells and multiple factors. There is much evidence to suggest that cell death is a key factor in the development and progression of atherosclerosis (Tabas 2005). Vascular endothelial cell death is an agonist of atherosclerosis. The death of smooth muscle cells can cause plaque instability and rupture, while the death of macrophages is beneficial to the stability of plaques. The development of atherosclerosis begins at the site of repeated endothelial tissue injury (endothelial cell dysfunction), with common features such as decreased levels of nitric oxide in the arterial wall and increased secretion of

endothelin-1 (ET1), angiotensin II (ANGII), and thromboxane. Reducing NO production may lead to endothelial cell apoptosis, and it has been demonstrated that upregulation of ANGII during senescence can prevent endothelial cell regeneration. In view of the close relationship between cell death and autophagy, the interactions between autophagy and atherosclerosis have recently been studied. The results show that the ultrastructure of intact autophagic components is found in atherosclerotic plaques. Autophagy is activated in all three major cells (vascular endothelial cells, smooth muscle cells, and macrophages) in atherosclerotic plaques. Therefore, autophagy plays an important role in the development of arteriosclerosis. In recent years, it has been found that the level of autophagy in atherosclerotic plaques is elevated and exhibits unique morphological features: myelin-like structure, cytoplasmic ubiquitination, inclusion body accumulation, and increased foam cells.

At present, the study of atherosclerosis is mainly carried out in mouse models. It was previously found that when Beclin-1 and ATG5 are knocked out, autophagy is inhibited, which exacerbates the progressive growth of atherosclerotic plaques. Apolipoprotein E (ApoE) is a protein that transports lipids. Apolipoprotein E knockout in mice fed a Western diet can lead to the development of arteriosclerosis, thus simulating an arteriosclerosis model in humans.

Atherosclerosis is the pathological basis of most cardiovascular diseases (CVD) and is closely related to cholesterol accumulation in the intima of arteries. Removal of excess cholesterol by the reverse cholesterol transport (RCT) pathway suggests a major anti-atherosclerotic mechanism. Reverse cholesterol transport (RCT), is a physiological process in which excess peripheral cholesterol is transported to the liver by HDLs and excreted into bile and feces. It is thought to be a key mechanism by which HDLs prevents atherosclerosis. However, knockdown of A TP-binding cassette transporter A1 (ABCA1), lecithin:cholesterol acyltransferase (LCAT), or apolipoprotein AI (apoA-I) in mice had no effect on neutral sterol content in feces, indicating the presence of an HDL-independent pathway that eliminates excess cholesterol in the body. The researchers then proposed a new cholesterol transport model that included not only the traditional RCT pathway but also additional steps, such as those for cholesterol absorption in the small intestine, cholesterol influx, and esterification in peripheral cells, LDL uptake in the liver, and transaminase cholesterol excretion (TICE) (Yu et al. 2018).

18.2.1 Autophagy and Atherosclerosis of Vascular Endothelial Cells

Vascular endothelial cells are a layer of flat cells that cover the entire inner surface of the cardiovascular system. The entire vascular endothelium of humans consists of approximately $1–6 \times 10^{13}$ endothelial cells that weigh approximately 1 kg. A large number of studies have shown that the endothelial layer is a highly metabolically active organ with very complex functions. Attached to the surface of endothelial

cells is a layer of glycocalyx (GCX), which is a major contributor to endothelial cell function and endothelial cell-dependent vascular health and is the first line of defense against cardiovascular diseases, including atherosclerosis, and GCX degradation. It can cause lipid deposition in blood vessel walls and is a marker of atherosclerosis. There is much evidence to suggest that some substances in the circulatory system or in the subendothelial layer of plaque can induce autophagy in vascular endothelial cells. For example, endostatin can induce autophagic death in human EA.hy926 endothelial cells. When EA.hy926 endothelial cells were treated with ox-LDL, which accumulates in the middle layer of plaque, the level of autophagy was enhanced compared with that after treatment with LDL or culture medium alone. Autophagy can preserve the function of endothelial cells by reducing oxidative stress, increasing the bioavailability of nitric oxide, and reducing vasculitis. Autophagy decreases in aged vascular tissue.

A variety of pro-atherogenic factors affect autophagy in vascular endothelial cells, indicating that autophagy in vascular endothelial cells is closely related to the onset of atherosclerosis. The study found that chemokines can upregulate the level of LC3-II in endothelial cells, promote autophagy-related gene 12 (Atg12), autophagy-related gene 7 (Atg7), and autophagy-related gene 5 (Atg5) expression, activate AMPK, inhibit the activity of mTOR, and promote the activation of ROS, indicating that chemokines influence autophagy in different ways (Martinet et al. 2013). Free fatty acids induce autophagy in vascular endothelial cells in a calcium-dependent manner by inhibiting mTOR activity through the adenosine monophosphate-activated protein kinase pathway. In the early stages of arteriosclerosis, moderate levels of autophagy can help endothelial cells respond to stress and thus promote cell survival (Xie et al. 2011); however, in the late stages of atherosclerosis development, under continuous stimulation by ox-LDL and inflammatory factors, endothelial cells die due to the excessive autophagy that also leads to plaque instability and rupture, greatly increasing the risk of thrombosis and the resulting acute coronary syndrome.

Phosphatidylcholine-specific phospholipase C (PC-PLC) can specifically hydrolyze phosphocholine to produce diacylglycerol (DAG) and phosphorylcholine (PC). As an important second messenger in the cell, DAG can regulate many cellular processes, such as cell metabolism, growth, differentiation, aging, and apoptosis. PC-PLC plays a key role in the apoptosis and aging of vascular endothelial cells. In vascular endothelial cells, the PC-PLC inhibitor D609 can also inhibit apoptosis induced by serum without growth factors. Studies have found that sphingosine phosphorylcholine and low concentrations of cadmium ions in vascular endothelial cells induce autophagy and that PC-PLC activity is significantly downregulated (Dong et al. 2009; Ge et al. 2011). Therefore, PC-PLC may be involved in the negative regulation of autophagy in vascular endothelial cells. Further studies have found that PC-PLC can interact with phosphatidylethanolamine-binding protein 1 (PEBP1) in endothelial cells, and PEBP1 can also negatively regulate autophagy in endothelial cells (Wang et al. 2013), indicating that both PC-PLC and PEBP1 play important roles in regulating the autophagy of endothelial cells. The results of in vivo experiments showed that the expression of PC-PLC in the atherosclerotic plaque in endothelial cells of ApoE-/- mice was increased, and the activity of PC-PLC in serum was also

significantly higher than that in the control group. D609 injected intraperitoneally further inhibited the activity of PC-PLC in ApoE-/- mice. It was found that D609 in ApoE-/- mice also significantly inhibited the development of atherosclerotic plaque, plaque lipids, and macrophage accumulation and preserved smooth muscle cells and collagen content, thereby enhancing the stability of plaque (Zhang et al. 2010). These findings reveal that PC-PLC is of key importance in autophagy in endothelial cells and as a factor in atherosclerosis.

Annexin A7 (ANXA7) binds to calcium ions and phospholipids to exert GTPase activity during the lipid membrane fusion process. Recent studies have found that a novel benzoxazine derivative, 2,3-dihydro-3-hydroxymethyl-6-amino-1,4-benzoxazine (ABO), promotes colocalization of ANXA7 and LC3. Decreased expression of ANXA7 protein significantly reduced the production of autophagosomes. This finding indicates that ABO promotes autophagy in vascular endothelial cells via ANXA7 (Wang et al. 2010). Other studies have found that ABO inhibits the GTPase activity of ANXA7 by targeting ANXA7, ANKA7-interacting protein grancalcin (GCA), and T cell intracellular antigen-1 (TIA1). The level of phosphorylation promotes autophagy in vascular endothelial cells (Li et al. 2013b; Huang et al. 2014). These findings suggest that ABO is a good protectant and inducer of autophagy in endothelial cells. ABO was injected intraperitoneally to examine its effect on atherosclerotic plaques in ApoE-/- mice. It was found that ABO can also inhibit autophagy of vascular endothelial cells and upregulate apoptosis of endothelial cells by upregulating ANXA7, which inhibited the development of atherosclerosis in ApoE-/- mice and improved the stability of plaques, indicating that ABO can be used as a treatment for arteriosclerosis. Likewise, ANXA7 has become a leading compound for therapeutic drug development as it may have anti-atherosclerotic effects (Li et al. 2013a). In addition, ABO can also promote the colocalization of ANXA7 and PC-PLC, reducing the level and activity of PC-PLC in endothelial cells in plaque and in serum. With the development of arteriosclerosis, the levels of PEBP1 in plaque endothelial cells and serum were significantly increased, and ABO significantly inhibited the increase. This finding indicates that the ANXA7/PC-PLC/PEBP1 signaling pathway plays an important role in associated autophagy and atherosclerosis. These studies have shown that promoting and maintaining autophagy in endothelial cells can inhibit the development of arteriosclerosis.

Early studies have shown that the butyrolactone derivative 3-benzyl-5-(2-nitrophenoxymethyl)-γ-butyrolactone (3BDO) regulates endothelial cell fate. Specifically, 3BDO significantly inhibited apoptosis of endothelial cells induced by serum and anti-growth factor. In this process, 3BDO did not significantly affect the level of ROS in cells but inhibited the expression of the membrane protein integrin β4. This study provided a new tool for exploring the role of integrin β4 in apoptosis and a theoretical basis for the treatment of cardiovascular disease (Wang et al. 2007a). Subsequent studies in endothelial cells have shown that with growth factor-free serum, apoptosis, and aging occurred simultaneously, and 3BDO can simultaneously inhibit cell aging and apoptosis induced by serum without growth factors. This finding on cell aging and apoptosis suggests the relationship between the two can be used a reference as it reveals that membrane integrin β4 may be an important correlative factor for

both apoptosis and aging (Wang et al. 2007b). In addition, 3BDO maintains endothelial cell migration and angiogenesis while inhibiting smooth muscle cell migration and proliferation, demonstrating that 3BDO is cell specific (Meng et al. 2008). In view of the close relationship between apoptosis, aging, and autophagy, the effect of 3BDO on autophagy in endothelial cells was further studied. The results showed that 3BDO can also protect endothelial cells from damage caused by different autophagic stimuli. For example, 3BDO inhibits LPS-induced autophagy damage by inhibiting the regulatory loop between nuclear protein 1 (NUPR1/p8) and the autophagic regulator p53 in endothelial cells. It also inhibits the accumulation of autophagic vesicles caused by chloroquine and disruption of the membrane potential in mitochondria (Huang et al. 2009).

Recent studies have shown that the direct target of 3BDO in endothelial cells is mTORC1, which inhibits autophagy by competitively binding to the 12-kDa FK506-binding protein 1A (FKBP12) (Di Ge1, 2014). The 3BDO-induced activation of mTORC1 inhibits the expression of long noncoding RNA TGFB2 overlap transcript 1 (TGFB2-OT1), an independent transcript derived from cleavage of the three prime untranslated region (3′-UTR) of transforming growth factor β2 (TGF-β2), through phosphorylation of TIA1. Specifically, TGFB2-OT1 1 binds to miR-4459, inhibiting the expression of autophagy-related gene 13 (Atg13) and thus promoting autophagy. 3BDO inhibits TGFB2 by activating mTORC1-TGFB2-OT1, which in turn reduces the protein level of Atg13 and thus inhibits autophagy. Subsequent studies have shown that 3BDO also inhibits production of inflammatory cytokines in vitro and in vivo. Autophagy and inflammation are very closely related processes, and autophagy can be induced and regulated by inflammatory responses. SQSTM1/P62 is a multifunctional scaffold protein involved in various processes, including signal transduction, cell proliferation, cell survival, death, inflammation, tumorigenesis, and oxidative stress. SQSTM1 is an autophagic substrate and is widely used as a marker for autophagic degradation but also acts as a scaffold for many proteins that promote effector protein interactions with substrates. The subsequent effector signal is then passed downstream to activate the NF-κB signaling pathway. Previous studies have shown that SQSTM1 also activates CASP1 and then increases IL1B levels. Lipopolysaccharide (LPS) and ox-LDL increased the level of TGFB2-OT1, which was inhibited by 3BDO. The lncRNA TGFB2-OT1 induced downregulation of MIR3960, MIR4488, and MIR4459 and increased levels of CERS1, NAT8L, ATG13, and LARP1 by isolating these miRNAs. LARP1 further increases the levels of ATG3, ATG7, and SQSTM1, and TGFB2-OT1 promotes autophagy via CERS1, NAT8L, ATG13, ATG3, and ATG7 and participates in inflammation by promoting SQSTM1 protein synthesis and activation of RELA and CASP1. Activations of LPS and ox-LDL via NUPR1 and TIA1 promote TGFB2-OT1 processing; TGFB2-OT1 is involved in autophagy and inflammation. 3BDO induces TIA1 phosphorylation via FKBP1A and MTORC1, of which the latter is part of TGFB2-OT1 processing system, and then, these 19 factors induce inflammation and autophagic TGFB2-OT1. TGFB2-OT1 is an attractive target for inflammation in VECs, and 3BDO may be a potential therapeutic compound for the development of new drugs targeting vascular disease (Huang et al. 2015).

As an important energy and metabolic regulator, mTOR is involved in the development of atherosclerotic plaques. Inhibition of mTOR activity in macrophages stabilizes plaques (Verheye et al. 2007), but inhibition of mTOR activity in endothelial cells leads to dysfunction and promotes thrombosis and cell death. While mTOR has a protective effect on angiogenesis and tissue regeneration, promoting the activation of mTOR may be an effective strategy for inhibiting or treating atherosclerosis. ox-LDL induces autophagy and apoptosis of vascular endothelial cells by inhibiting mTOR, which in turn promotes arteriosclerosis. Therefore, 3BDO inhibits vascular endothelial cell function by inhibiting the effects of ox-LDL on mTOR, thereby inhibiting atherosclerosis. In addition, in macrophages (RAW246.7) and vascular smooth muscle cells, 3BDO did not affect changes in mTOR activity or autophagy caused by ox-LDL. In human vascular endothelial cells cultured in vitro and ApoE-/- mouse arterial endothelium, 3BDO not only inhibited ox-LDL-induced autophagy but also inhibited the apoptosis it induces, thereby controlling the development of atherosclerosis, and at the same time, the inflammatory response was significantly suppressed. In summary, in vascular endothelial cells, ox-LDL-induced mTORC1 activity is decreased, which promotes the development of atherosclerosis. First, the decrease in mTORC1 activity promotes the production of TGFB2-OT1, which further upregulates the level of ATG13, promoting the autophagy and endothelial cell apoptosis caused by autophagic damage, plaque instability, and atherosclerosis. As an agonist of mTORC1, 3BDO inhibits the decrease in mTORC1 activity caused by ox-LDL, thereby promoting the stability of plaque and inhibiting the development of atherosclerosis.

Some studies have shown that, in atherosclerosis, some microRNAs regulate autophagy and thus affect the progression of the disease. For example, in the latest research, microRNA-155 was found to inhibit Rheb/mTOR signaling in endothelial cells. The pathway promotes autophagy; therefore, microRNA-155 acts as a regulator of endothelial cell function under ox-LDL stress, making it a potential candidate for new therapeutic strategies against atherosclerotic disease.

These studies demonstrate that inhibition of ox-LDL-induced autophagy in endothelial cells can inhibit the development of arteriosclerosis. Microparticles (MPs) are small membrane vesicles that are released by various cells under physiological and pathological conditions. In the past, these particles were considered to be inert cell debris, but many recent studies have shown that they may play a role in intercellular communication. It has been reported that MP levels are increased under various pathological conditions, including infections, development of malignant tumors, and autoimmune diseases such as rheumatoid arthritis (RA). RA is a systemic inflammatory disease characterized by chronic synovial inflammation that causes cartilage and bone damage, accelerates atherosclerosis, and increases mortality. According to the literature, MPs may also play a role in the dysfunction of endothelial cells, leading to atherosclerosis in RA patients. Autophagy is a repair process by which cytoplasmic components are sequestered in double-membrane vesicles, which are subsequently degraded upon fusion to a lysosomal compartment. Therefore, MPs may lead to an atherosclerotic process through the dysregulation of autophagy in endothelial cells.

In a recent study, SIRT1 was found to be a human ortholog of the yeast protein silencing information regulator 2 and is a nicotinamide adenine dinucleotide-dependent histone deacetylase. Convincing evidence suggests that SIRT1 plays a key role in a variety of biological processes, including cellular metabolism, stress response, inflammation, autophagy, and apoptosis. Studies have shown that SIRT1 inactivation induces inflammation through NF-κB activation and autophagy dysregulation in THP-1 cells. Endothelial cell-specific SIRT1 has been shown to be a true anti-atherosclerotic factor by improving endothelial cell survival and function. By inhibiting the migration of foam cells derived from vascular smooth muscle cells, SIRT1 is considered to be a promising new intervention target for AS and induces autophagy. Recent studies have shown that inhibition of SIRT1 by EX-527 reverses increases in the LC3-II/LC3-I ratio and decreases in p62 levels that were induced by ox-LDL in HUVECs, indicating that inhibition of SIRT1 inhibits ox-LDL-induced autophagy in HUVECs. In addition, EX-527 (SIRT1 inhibitor) abrogated the promotion of ox-LDL-induced autophagy by Paeoniflorin (Pae) in HUVECs, indicating that Pae enhances ox-LDL-induced activation of autophagy by upregulating SIRT1 expression. Similarly, resveratrol has previously been reported to enhance ox-LDL-induced autophagic flux in HUVECs by upregulating SIRT1. It was also found that inhibition of SIRT1 expression restored the inhibitory effect of Pae on ox-LDL-induced apoptosis and adhesion molecule expression. Taken together, these results indicate that Pae attenuates ox-LDL-induced apoptosis and adhesion molecule expression by upregulating SIRT1 in HUVECs (Wang et al. 2018a) (Fig. 18.1).

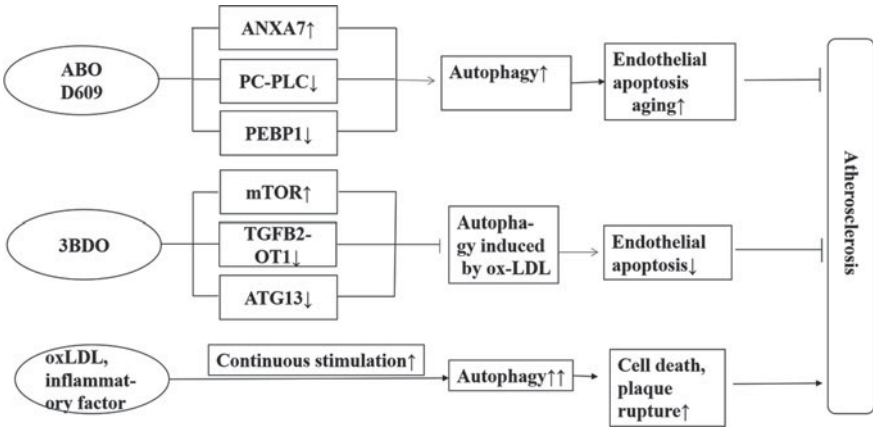

Fig. 18.1 Autophagy and atherosclerosis of vascular endothelial cells:ABO, D609 targeting ANXA7, PC-PLC, and PEBP1 activates autophagy, then promotes apoptosis and aging of endothelial cells and inhibits atherosclerosis; 3BDO targets mTOR, TGFB2-OT1, and ATG 13 and regulates ox-LDL-induced autophagy. Then, it inhibits endothelial cell apoptosis and atherosclerosis; ox-LDL inflammatory factor continuously promotes cell death and plaque rupture to regulate atherosclerosis via activation of autophagy

18.2.2 Autophagy and Atherosclerosis of Smooth Muscle Cells

The morphological characteristics of typical autophagy were observed by transmission electron microscopy when observing the smooth muscle cells isolated from the fibrous cap of late-growing plaque, including the formation of myelin-like structures, the accumulation of ubiquitinated components, and an obvious cavitation phenomenon. Myelin-like structures include phospholipids and membrane fragments that often appear in concentric rings that represent autophagic degradation of cell membrane components. These structures appear not only in human plaques but also in plaques of cholesterol-fed rabbits (Schrijvers et al. 2011). The ubiquitinated component accumulates in the smooth muscle cytoplasm, which shows signs of autophagic death in the fibrous cap. Studies have shown that the autophagy-inducing 7-ketocholesterol can promote vacuolation of cultured smooth muscle cells, ubiquitination of proteins and expression levels of LC3-II. In smooth muscle cells isolated from atherosclerotic plaques, stimulation of the inflammatory factor tumor necrosis factor-α (TNFα) promotes the number of vacuolar cells, activates LC3-II and upregulates expression of autophagy-related gene 6 (Atg6/Beclin1). Studies have shown that treatment with 7-ketocholesterol can inhibit the death of smooth muscle cells induced by low concentrations of statins. The mechanism may be initiated by damaged organelles cleared by autophagy to limits the release of mitochondrial pro-apoptotic proteins such as cytochrome c. Furthermore, excess free cholesterol or the lipid superoxide product 4-hydroxydecanoic acid induces the development of autophagy in smooth muscle cells, which in turn promotes smooth muscle survival (Xu et al. 2010). These results indicate that appropriate levels of autophagy in smooth muscle cells can protect plaques from oxidative stress by degrading damaged intracellular material, especially depolarized mitochondria. Autophagy is an evolutionarily conserved mechanism that is linked to a variety of cellular pathways that affect the survival and function of vascular smooth muscle cells (VSMCs). Activation of autophagy by intercellular and/or extracellular stimulation has a protective effect against VSMC against cell death, whereas overactivated autophagy is considered to be a deleterious process that results in excessive self-digestion. Changes in autophagy have been documented in VSMCs in response to various stimuli, leading to modulation of VSMC function, including proliferation, migration, matrix secretion, contraction/increased diastolic pressure, and differentiation. Every change in VSMC plays a key role in the development of vascular disease. Importantly, emerging evidence suggests that autophagic defects in VSMCs lead to atherosclerosis and restenosis, providing a new perspective for development of therapeutic targets against vascular disease (Fig. 18.2).

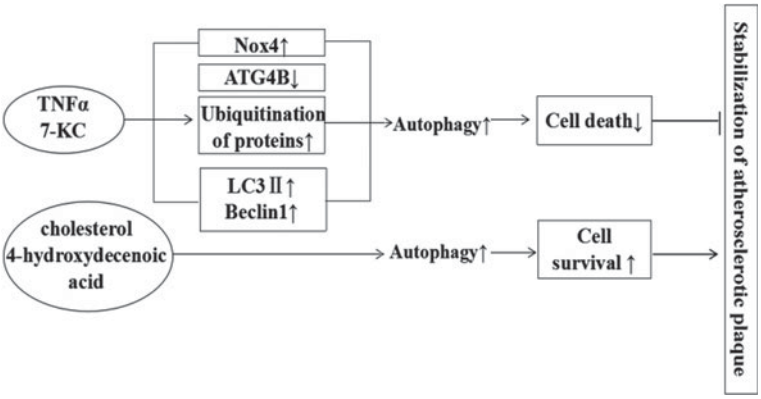

Fig. 18.2 Autophagy and atherosclerosis of smooth muscle cells: TNFα 7-KC inhibits cell death and destroys the stability of atherosclerotic plaques by targeting Nox4, ATG4B, and other factors and activating autophagy. Cholesterol 4-hydroxydecenoic acid activates autophagy to promote cell survival and stabilize atherosclerotic plaques

18.2.3 Autophagy and Atherosclerosis of Macrophages

Recent studies have demonstrated that autophagy of macrophages is an important way to promote vascular pathology. Stabilin-1 (STAB1) is a scavenger receptor expressed on alternately activated macrophages and sinusoidal endothelial cells. Its ligands include ox-LDL and extracellular matrix glycoprotein SPARC, which are present in human and mouse atherosclerotic lesions. However, recent studies have shown that bone marrow-specific Stabilin-1 deletion does not affect the susceptibility of mice to atherosclerosis. Because macrophages have a relatively strong phagocytic function, it is difficult to observe the structure of the vesicles in cells by electron microscopy because it is impossible to determine whether the structure is undergoing autophagy (Schrijvers et al. 2011). The observation of autophagy marker protein LC3 by immunoelectron microscopy is a more specific method for detecting autophagy, but it is also difficult to see the macrophages because the expression level of LC3 is very low in these cells. Furthermore, in macrophages, lysosomal marker proteins such as cathepsins are highly expressed, and therefore, it is very easy to produce false positives when these enzymes are detected by immunohistochemistry. Recent studies have shown that macrophage phagocytosis of sterols (such as β-sitosterol) causes cells to die in a caspase-independent manner that involves autophagy. In most people, plasma and arteriosclerotic plaques contain very low levels of sterols, but in patients with excess glutamine, plasma levels of phytosterols reach very high levels, leading to severe atherosclerosis. Phytosterols are substrates for acyl-CoA: cholesterol acyltransferase (ACAT), a sterol esterase that inhibits cell death by inhibiting the insertion of free sterols into cell membranes (Liu et al. 2005). Therefore, free phytosterols can accumulate in plaque macrophages, causing autophagic death, lesion necrosis, and plaque instability in macrophages.

Recently, studies have found that autophagy of macrophages has a protective effect on the development of atherosclerosis. Using ApoE-/- mice as a model for studying atherosclerosis, it was found that autophagy markers p62 and LC3 were mostly colocalized with leukocytes and macrophages in plaques. With the development of atherosclerosis, autophagy is gradually reduced, which can be found by detecting the accumulation of p62 in plaques. Loss of autophagy in macrophages promotes the development of atherosclerosis in ApoE-/- mice, hyperactivation of macrophage inflammatory bodies, and overproduction of interleukin 1β (IL-1β). Other studies have shown that MKP-1 deficiency in monocytes and macrophages accelerates atherosclerosis and the progression of lesions by reducing autophagy, enhancing apoptosis, and regulating macrophage polarization, whereas MKP-1-induced overexpression protects macrophages from dysfunction caused by metabolic stress. Therefore, it is speculated that MKP-1 is a major regulator of macrophage function and fate.

Recent studies suggested that a natural sugar called trehalose can act as an inducer of autophagy–lysosomal biogenesis in macrophages and showed that trehalose promotes macrophage autophagy, the major transcriptional regulator of lysosomal biogenesis (TFEB) overexpression, thereby relieving atherosclerotic properties.

Another important aspect is to consider is the role of lipid autophagy in vascular pathology. Lipid autophagy is a special type of autophagy that promotes the conversion of cholesterol from adipose cells to HDLs through the action of lysosomal lipases (Liu and Czaja 2013a). Autophagy promotes the hydrolysis of cholesterol lipid droplets stored in macrophages, thereby promoting the outflow of cholesterol. Studies have found that phosphatase (Wip1) and serine/threonine kinase (Atm) negative regulators of signaling pathways play an important role in autophagy and cholesterol efflux in ApoE-/- mice. The loss of Wip1 inhibits the conversion of macrophages into foam cells, thereby inhibiting the formation of atherosclerotic plaques (Le Guezennec et al. 2012).

In conclusion, in atherosclerosis, the autophagic function of macrophages is dysregulated, and the defect of macrophage autophagy promotes vasculitis, oxidative stress and plaque necrosis, indicating that autophagy controls macrophages for effective inhibition (Fig. 18.3).

18.2.4 The Role of Autophagy in Atherosclerosis

There are a variety of autophagy inducers in atherosclerotic plaques, including the accumulation of inflammatory factors, ROS, and ox-LDL. Since autophagy is thought to be a mechanism of survival rather than a signal to promote death, it can be speculated that autophagy of smooth muscle cells in the fibrous cap of advanced plaque is an important mechanism for plaque stabilization. In fact, autophagy is more likely to protect plaque cells against oxidative stress, which is a major feature of late-forming

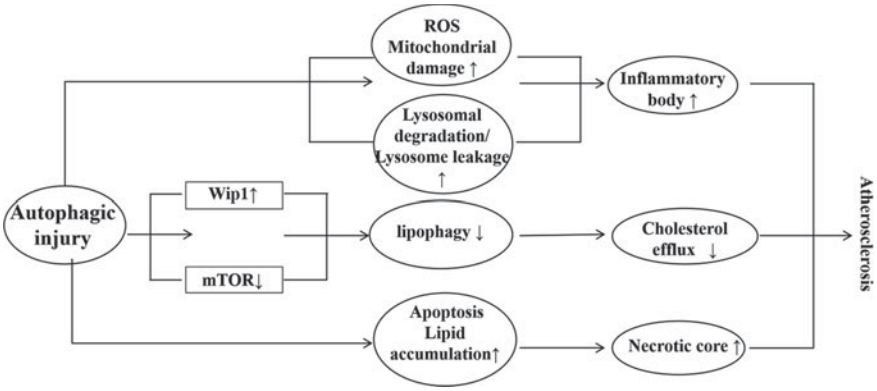

Fig. 18.3 Autophagy and atherosclerosis of macrophages: Autophagic damage in macrophages affects atherosclerosis in three ways: (1) excessive accumulation of ROS and lysosomal damage, hyperactivation of macrophage inflammatory bodies and IL-1β promotion of atherosclerosis development in ApoE-/- mice; (2) changes in Wip1 and mTOR levels in autophagic damaged cells reduce lipid autophagy and cholesterol efflux, and accelerate atherosclerosis development; and (3) autophagy damage leads to lipid accumulation, promotes the formation of a necrotic core, and accelerates atherosclerosis

plaques, thereby degrading damaged components, specifically before cytochrome C is released. Thus, autophagy of damaged substances is a nonapoptotic way to promote cell repair. However, the presence of acute or sustained oxidative stress can result in an increase in intracellular levels of ROS that damage the lysosomal membrane. Changes in mitochondrial components inhibit membrane fusion with the autophagic vesicles containing the damaging substance and result in the release of potent hydrolytic enzymes, thereby exacerbating the extent of cellular damage. If autophagy is not involved in the oxidative stress response of atherosclerotic plaques or if oxidative damage exceeds the ability of a cell to prevent it, cells will die through by apoptosis.

However, unlike baseline autophagy, excessive levels of autophagy may lead to autophagic death of smooth muscle cells, which reduces collagen synthesis, leading to thinning of the fibrous cap and plaque. Moreover, autophagic death of endothelial cells is also detrimental to the structure of plaques, as endothelial cell damage or death is the primary mechanism leading to plaque thrombosis in acute clinical events. Due to the important role of macrophages in atherosclerosis and plaque instability, autophagic death of macrophages is an effective means of stabilizing nonobstructive unruptured plaques.

In conclusion, autophagy is a ubiquitous stress response in vascular cells and plays an important role in promoting plaque stabilization. However, from the perspective of plaque stability, excessive autophagy is harmful. In atherosclerotic plaques, moderate autophagy can maintain plaque stability. In recent years, researchers have focused on the study of autophagic defects, which occur mainly through two different types of situations. In one case, defects occur during the initial period of autophagy, which

mainly prevents the formation of autophagic vesicles; in the other case, defects occur during fusion with lysosomes or degradation of the lysosomal pathway. Currently, the defect in the degradation process of the lysosomal-dependent pathway is considered most common. Studies have shown that autophagic defects in vascular smooth muscle cells accelerate aging and promote intimal formation and atherosclerosis.

Basal levels of autophagy can promote cell survival and maintain plaque stability in atherosclerosis, but excessive autophagy can promote cell death, which can lead to severe cardiovascular and cerebrovascular diseases. When vascular smooth muscle cells die, the amount of secreted collagen is reduced, which causes the fibrous cap to become thinner and thereby rupturs the plaque. Studies have shown that excessive autophagy in smooth muscle cells leads to their death, making them more susceptible to plaque formation. A large number of studies have shown that cell death caused by autophagy will induce the release of many inflammatory factors, such as IL-1β, IL-6, and TNF-α, which in turn cause an inflammatory response. Severe oxidative stress and autophagy form a wax-like complex of a protein and an oxidized lipid. This wax-like body cannot be removed by lysosomal hydrolase and attracts a large amount of lysosomal enzymes for autophagy. The binding of the body to the lysosome is limited, leading to cell death. Thus, autophagic death of cells promotes the formation of unstable plaques (Nussenzweig et al. 2015).

18.2.5 Application of Drugs Targeting Autophagy in Atherosclerosis

In the past decade, many new or existing drugs have been discovered in vitro to promote or inhibit autophagy, especially in tumors and neurodegenerative diseases. In the latest research, the treatment of atherosclerosis by targeted autophagy has led to a new direction of clinical treatment. The main drugs currently used for the treatment of atherosclerosis are everolimus, a white gourd alcohol, and berberine, which inhibit the autophagy inhibitor mTOR. In addition, berberine can activate AMPK. β-Arrestin-1 overexpression, which along with CB2R activation, is thought to increase the expression of Beclin1 and LC3, thereby promoting the occurrence of autophagy (Shao et al. 2016). Currently, autophagy is mainly regulated through the classical mTOR signaling pathway, of which mTOR is an energy-sensing kinase that plays an important role in regulating protein synthesis, cell growth, and metabolism. For example, rapamycin and its derivatives can inhibit the activity of mTOR to activate autophagy. Targeting mTOR-independent signaling pathways, primarily through the level of inositol triphosphate, can also be used to regulate autophagy. This process can be adjusted by some drugs, such as sodium valproate and carbamazepine. It should be noted that changes in autophagy by drugs may have other effects, as these drugs usually block some routine processes such as glucose metabolism and those of the mitochondrial respiratory system. Broad-spectrum inhibitors of PI3K, such as wortmannin and 3-methyladenine (3-methyladenine, 3-MA), are widely used to

inhibit autophagy and to inhibit protein kinase Akt. Currently, there are no specific inhibitors of autophagy. Inhibition of autophagy-related gene 4 (Atg4) tends to significantly inhibit autophagy. Since Atg4-specific substrates have been recently found (Shu et al. 2010), studies are being conducted to design Atg4-specific inhibitors. It is an effective method for blocking autophagy.

Recent studies have shown that nitrosamines can reduce lipid accumulation and necrotic core formation in atherosclerotic plaques by inducing autophagy; another study suggested that puerarin is a main bioactive isoflavone compound extracted from the traditional Chinese herbal medicine Pueraria lobata, which is clinically used for the treatment of cardiovascular diseases, hypertension and diabetes in China and other Asian nations. ERK5/KLF2 activation is involved in the adhesion of puerarin to endothelial cells and the reduced effects of atherosclerotic lesions in ApoE-/- mice.

It is well known that angiogenesis and vascular remodeling are defensive mechanisms invoked during ischemic cardiovascular events, including peripheral arterial disease (PAD) and myocardial infarction, to restore blood supply and oxygenation in tissue; the endothelium plays a key role in these intrinsic protection processes. Recent studies have shown that C-type natriuretic peptide (CNP) is a basic signaling factor in endothelial cells that coordinates vascular homeostasis. Clinical vascular ischemia is associated with decreased levels of CNP and its homolog NPR-C.

18.2.6 Conclusion

Current research indicates a very close and complex relationship between autophagy and hyperlipidemia. The difference in intracellular lipid content affects the level of autophagy in the cells. Short-term lipid stimulation can significantly promote autophagy, but prolonged or specific types of lipid stimulation can inhibit autophagy levels. Studies have shown that hyperlipidemia caused by aging and high-fat feeding is often accompanied by lower levels of autophagy in vivo. Autophagy regulates lipid metabolism by means of lipid autophagy, which in turn affects intracellular cholesterol and triacylglycerol levels. Induction of autophagy can significantly reduce lipid content in cells and mice. Key autophagy factors such as Atg14, mTOR, and LC3 may play important roles in the relationship between autophagy and hyperlipidemia. However, the specific molecular mechanisms associated with autophagy and hyperlipidemia remain to be further studied (Fig. 18.4).

Based on the current findings, it can be determined that cells require autophagy to promote survival under stress conditions. Therefore, moderate regulation of autophagy is an effective strategy for stabilizing atherosclerotic plaques. The challenge in the future for stabilizing atherosclerotic plaques involves ways to selectively protect autophagy to promote survival without causing unnecessary death or pro-inflammatory responses. Applications of infection-resistant nanoparticles in tissue-specific drug-targeted therapies prove useful for specifically inducing autophagy in macrophages and stabilizing atherosclerotic plaques. However, stimulation of

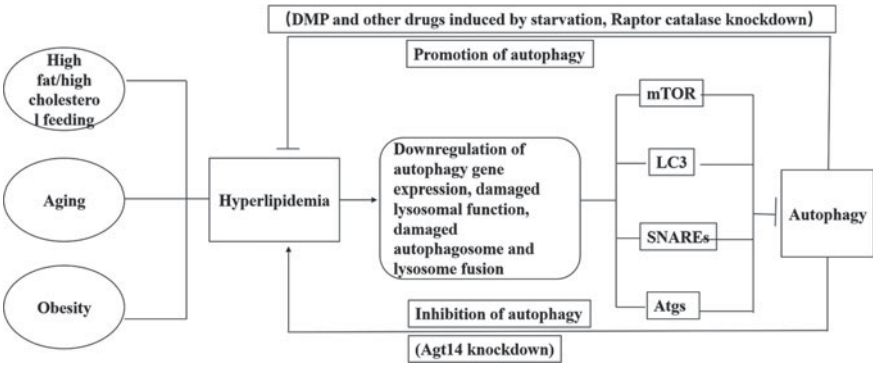

Fig. 18.4 Relationship between autophagy and hyperlipidemia: Hyperlipidemia caused by aging and high-fat feeding is often accompanied by a lowered level of autophagy. Autophagy regulates lipid metabolism by means of lipid autophagy, which in turn affects intracellular cholesterol and triacylglycerol levels and induces autophagy. Autophagy can significantly reduce the lipid content in cells. Key factors such as Atg14, mTOR, and LC3 may play important roles in the relationship between autophagy and hyperlipidemia

autophagy is only beneficial if the regulatory mechanisms remain intact because excessive autophagic activity leads to the efflux of lysosomes or autophagosomes, as well as cell death. Studies have shown that histone deacetylase plays a role in autophagy and arteriosclerosis. For example, inhibition of histone deacetylase 1 can promote autophagy and reduce neointimal formation in mice with vascular injury. Due to the broad-spectrum effect of histone deacetylase and lower side effects, it may become a new target for the treatment of atherosclerosis through regulation of autophagy, but there are still many unresolved issues, for example, how long does autophagy activation last before it damages cells? Does autophagy in plaques affect other mechanisms of cell death? Does autophagy induction create resistance to the development of atherosclerosis? These issues require further research.

In conclusion, autophagy can be thought of as a cell response for survival. It can be adaptively countered by placing stress on the cells. If the response, such as atherosclerotic plaque, is more moderate, the oxidative damage is moderate. In this case, autophagy is activated to enable cells survival such that damaged, harmful, and unwanted cellular components are easily removed. On the other hand, if the stress on the cell is too high or the persistence of it is too long, adaptive responses will be effectively countered, and the cell will move toward a pathway of autophagic death. The exact mechanism of autophagic action in hyperlipidemia and atherosclerosis requires further investigation to determine whether the components of that autophagy are effective targets for the treatment of both diseases.

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Chapter 19
Application of Autophagy in Cardiovascular Diseases

Jie Du, Yulin Li, and Congcong Zhang

Abstract Autophagy is closely related to the pathogenesis and progression of cardiovascular diseases. Autophagy may be a therapeutic target for many cardiovascular diseases. In this chapter, we will summarize autophagy activators and inhibitors as potential drugs for cardiovascular diseases.

Keywords Autophagy inhibitors · Autophagy activators · Drug

Autophagy regulation is closely associated with cardiac diseases, including hypertension, cardiac hypertrophy, ischemic heart disease, IR injury, heart failure, and cardiomyopathy. Drugs that target autophagy are divided into two types: autophagy activators and autophagy inhibitors (summarized in Table 19.1 according to the mechanism) (Cheng et al. 2013; Towers and Thorbum 2016).

Under cardiac pressure overload, appropriate pressure activates cell autophagy, alleviates ER stress, and maintains mitochondrial function and cardiac function, thus exerting a protective effect. Inhibition of cardiomyocyte autophagy under physiological stress significantly aggravates the occurrence of heart failure. In contrast, under severe pressure overload, Beclin 1 upregulation induces autophagy overactivation, leading to loss of important organelles/proteins and cell death and rapid transfer from cardiac compensatory myocardial hypertrophy into decompensated heart failure. Under light and moderate pressure loads, low-dose AMPK agonists (such as AICAR and metformin) and mTOR inhibitors (rapamycin) appropriately activated autophagy, limited the occurrence of myocardial hypertrophy, improved cardiac function, and delayed the occurrence of heart failure (Li et al. 2014). As a SIRT1 agonist, resveratrol activates the FOXO1-RAB7 signaling pathway and promotes autophagy by SIRT1 or AMPK activation (Wang et al. 2014), which exerts a protective effect against pressure overload myocardial hypertrophy and heart failure. However, autophagy should be inhibited under severe overload. Trichostatin A (Type I-II histone deacetylase inhibitor) inhibits cellular autophagy. Under severe pressure overload, trichostatin A can block left ventricular pathological remodeling, which may be attributed to autophagosome squeezing (Cao et al. 2011). Inhibition

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Table 19.1 Classification of drugs that target autophagy

Classification	Drug name	Targets
Activators of autophagy		
mTOR inhibitors	Rapamycin, everolimus, sirolimus, perhexiamine iodine, niclosamide, gamalin, berberine	FKB12 binding Inhibit mTOR activation
AMPK activators	AICAR, metformin	Inhibit mTORC1/C2, activate ULK1
SIRT1 activators	Resveratrol, SRT1720, SRT2183	Deacetylate autophagy regulators
IP3 inhibitors	Carbamine, lithium salts, valproic acid	Downregulate intracellular calcium
Intracellular calcium inhibitors	Verapamil, amiodarone, nitrendipine	Downregulate intracellular calcium
cAMP inhibitors	Clonidine, ilmenidine	Inhibit PKA activation
ULK1 activators	L YN-1604	Activate ULK1
Chemicals	Trehalose	Unknown
Inhibitors of autophagy		
AMPK inhibitors	Compound C, ARA-A	Activate mTOR, inhibit ULK1
GSK-3 inhibitors	SB216763	Inhibit TSC2
Phagocytic inhibitors	Trimethyladenine	Inhibit PI3K
ULK1inhibitors	SBI-0206965	Inhibit ULK1

of Type I histone deacetylases can inhibit mTOR activity, which not only activates autophagosome formation but also promotes mature and scavenging. Thus, based on the physiopathological conditions of the heart, appropriate autophagy activating or inhibiting drugs should be selected to protect cardiac function (Fig. 19.1). In cardiomyopathy caused by protein misfolding, autophagy protects the heart by scavenging these proteins. In cardiomyopathy caused by toxic proteins attributed to the *CryAB* mutation, ATG7-dependent autophagy activation is attenuated, and multiple amyloid protein oligomers accumulate in cardiomyocytes. Deletion of the *Beclin-1* gene inhibits autophagy and accelerates cardiac function failure in mice with the *CryAB* mutation. In neurodegenerative diseases (such as Alzheimer's disease, Parkinson's disease, and Huntington's disease), mTOR inhibitors, AMPK activators, SITR1 activators, and trehalose exert their therapeutic effects via taking up and scavenging abnormally activated or aggregated proteins in cells, suggesting that these drugs can be applied in the treatment of protein-toxic cardiomyopathy. Carbamazepine and lithium treatment induces autophagy and promotes the accumulation of misfolded intracellular proteins in protein-toxic cardiomyopathy. However, this treatment's side effects limit the applications of such drugs (Fig. 19.1).

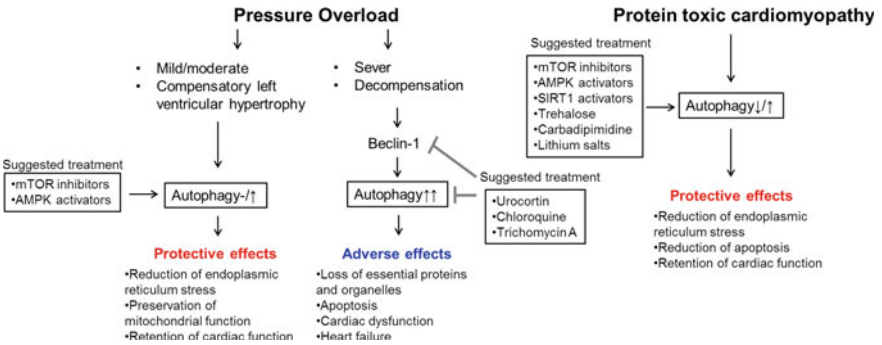

Fig. 19.1 Therapies targeting autophagy for pressure overload-induced cardiac diseases and protein toxic cardiomyopathy

Autophagy is activated in cardiac IR, but the influence of autophagy activation on pathological changes is different. During chronic/long-term ischemia, autophagy activation has a protective effect. Autophagy is upregulated in the hibernating myocardium of pigs, and the autophagy level in the ischemic area negatively correlates with apoptosis, suggesting that autophagy activation may inhibit cell apoptosis. The mechanisms of autophagy inhibition (by inhibiting endogenous AMPK or GSK and activating mTOR) and cardiac ischemic injury aggravation include the following: (1) autophagy promotes regeneration of amino acids and fatty acids, promotes ATP synthesis and compensates for energy loss and (2) autophagy scavenges damaged mitochondria and abnormally aggregated proteins, and protects cell function. In contrast, autophagy plays a harmful role in reperfusion injury. In reperfusion injury, oxidative stress upregulates Beclin 1 expression and promotes the formation of autophagic vacuoles. Downregulation of Beclin-1 blocks autophagy and reduces reperfusion injury, indicating that upregulated autophagy aggravates injury. The mechanism of GSK inhibition for blocking the occurrence of reperfusion injury is mTOR activation (Zhai et al. 2011). Urocortin inhibition of Beclin-1 can decrease cardiomyocyte death mediated by hypoxia-reoxygenation. However, under certain conditions, autophagy activation in reperfusion injury has a protective effect, although the reason is unclear. Extending the ischemia time reduces the protective effect of autophagy inhibition on reperfusion injury. Thus, we selected autophagy drugs based on pathological changes in the patients (ischemia or reperfusion injury). For example, patients with chronic stable coronary heart disease have stable subendocardial myocardial ischemia without requiring mechanical coronary artery recanalization. These patients can be given autophagy activators. However, patients with an acute and completely occluded coronary artery should be given autophagy activators in the acute ischemia stage, followed by autophagy inhibitors in the reperfusion stage. Unfortunately, the current evidence has not indicated the best timing. Note that molecules with ischemic changes participating in autophagy regulation, but not in the reperfusion injury pathological process, can be selected as a target. For example, AMPK and GSK in cardiac ischemia activate autophagy but do not participate

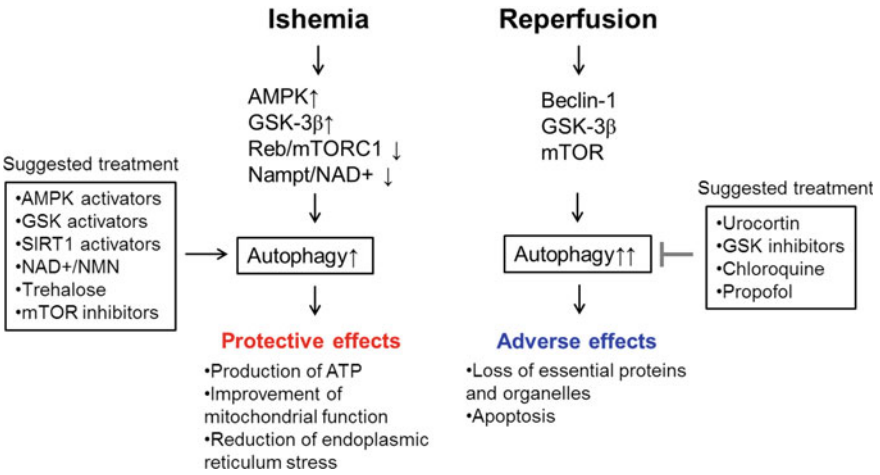

Fig. 19.2 Therapies targeting autophagy for cardiac ischemia/reperfusion injury

in the regulation of autophagy during the reperfusion process. Based on this strategy, aiming at signal transduction mechanisms at specific stages, AMPK and GSK activators are used to treat ischemic heart disease. Moreover, nicotinamide phosphoribosyltransferase (NAMPT) may be a therapeutic autophagy target. NAMPT has been shown to increase nicotinamide adenine dinucleotide (NAD^+) and ATP contents in cardiomyocytes, inhibit apoptosis, and promote autophagic flux in cardiomyocytes. Activated NAMPT induces cellular autophagy and inhibits myocardial ischemic injury. NAMPT and NAD^+ activate SIRT1 and further promote autophagy (Fig. 19.2).

Currently, promotion or inhibition of autophagy in the treatment of cardiovascular diseases is a complicated problem that lacks autophagy-specific drugs. However, some drugs developed for other therapeutic targets have been demonstrated to have regulatory effects on autophagy (Table 19.2), which may provide important insight into the treatment of cardiovascular diseases (Valentim et al. 2006; Salabei and Conklin 2013; Salabei et al. 2012; Xie et al. 2011).

Finally, autophagy as a therapeutic target is limited due to the following aspects: the pathogenesis of autophagy is not fully understood, which may influence drug specificity and limit effective candidate treatment. Autophagy drugs are beneficial against some diseases (infectious disease or neuropathy). Sirolimus, an approved mTOR inhibitor in the clinic, has been applied to enhance autophagy in an experimental model. The effectiveness of autophagy in diseases as a target is still being tested in current clinical trials. Many autophagy proteins and inhibitors can independently affect the biological process of autophagy activation, which makes drug design more complicated. However, understanding the mechanism of disease prevention by autophagy is continuously improving, which may lead to the discovery of

Table 19.2 Potential application of autophagy-regulating drugs in cardiovascular diseases

Drugs	Autophagy regulation	Potential applications
Verapamil	Activation	Atherosclerosis, vascular restenosis
Rapamycin	Inhibition	Myocardial infarction
Metformin	Activation	Heart failure, diabetic cardiomyopathy
Isoproterenol	activation	cardiac fibrosis
Doxorubicin	Activation	Cardiac hypertrophy
Paclitaxel	Inhibition	Myocardial infarction
Granulocyte colony-stimulating factor	Inhibition	Heart failure
Urocortin	Inhibition	Ischemic heart disease and heart failure
Berberine	Activation	Cardiac hypertrophy

new diagnostic and treatment targets. Screening of autophagy inhibitors and antagonists (including upstream regulatory factors and downstream targets of autophagy) may help find extra therapeutic targets.

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Part III
Autophagy and Cancer

Currently, cancer is the leading cause of death in developed countries and the second in developing countries. Further deepening the understanding of tumorigenesis is critical for the struggle against this severe public health problem. Tumorigenesis is a process in which normal cells undergo numerous genetic and epigenetic mutations under various stresses, gradually forming cancer cells and eventually developing into tumors. This process involves changes in many cellular intrinsic mechanisms and in the microenvironment. Understanding of the process is important for preventing tumorigenesis and recurrence. Numerous studies have shown that autophagy has always played a role in protecting cells throughout the tumor stage, but it mainly plays a different role in tumor suppression and cancer promotion in the early and late stages. This is mainly related to the protected object. Autophagy in the early stage of tumorigenesis is more important by inhibiting inflammation, maintaining cell genomic stability, promoting damaged cell senescence, and inhibiting the accumulation of p62, protecting normal cells from stability and inhibiting their transformation into tumor cells. In the late stage of tumorigenesis, the initial tumor cells have formed. The initial tumor cells have grown and transformed in a relatively infertile environment. In this process, autophagy mainly promotes tumor cell survival and proliferation through resisting internal and external stress and induces the vigorous metabolism of tumor cells.

Cancer stem cells (CSCs) have the potential of self-renewal, proliferation, and differentiation, and plays an important role in the process of tumorigenesis and progression. Existing studies have shown that autophagy plays an important role in tumor stem cell survival and invasion and metastasis, and autophagy has different effects on tumor stem cells at different stages of tumorigenesis and development. In the early stage of carcinogenesis and prior to induction of malignant tumor, autophagy can inhibit the formation of tumor stem cells by eliminating damaged organelles and proteins, maintaining genomic stability. In the late stage of tumor development, autophagy can provide protection for cancer stem cells in the infertile environment such as ischemia and hypoxia, promote their survival, and also inhibit tumor stem cell apoptosis.

A variety of mechanisms are involved in regulating tumor invasion and metastasis and interacting with each other, which form a large regulatory system. A large amount of evidence has shown that autophagy is also involved in the development of tumors including invasion and metastasis. Autophagy not only controls some biological of tumor cells, but also is affected by the microenvironment. The evidence suggest that autophagy can promote tumor invasion and metastasis; however, there is also evidence to support the inhibition of tumor invasion and metastasis by autophagy. Autophagy promotes tumor invasion through enhancing tumor stem cell phenotype, regulating cell adhesion kinetics, inducing epithelial–mesenchymal transition, inhibiting apoptosis caused by anoikis during metastasis, and providing the energy for tumor cell invasion.

With the continuous development of autophagy research, autophagy is involved in more and more cancer therapy. Autophagy plays a key role in promoting and inhibiting therapies. Chemotherapy is an important means of treating malignant tumors. The main role of chemotherapy drugs is to induce cell death. With the deepening of research, autophagy is inextricably linked with the occurrence of tumors, playing the role of "double-edged sword" in the process of chemotherapy. Whether chemotherapy drugs induce cell survival or death depends mainly on the type of tumor cells, the degree of differentiation, and the types of chemotherapy drugs. The mechanism by which autophagy switches between tumor cell survival and death remains unclear; the correlation between autophagy and tumor stem cells, tumor microenvironment, and detailed mechanisms remains to be elucidated. Studying the relationship between tumor chemotherapy and autophagy, the relationship between autophagy and apoptosis, and the regulation of autophagic death-related expression genes have important clinical significance for antitumor therapy. In addition, existing research has shown good application prospects in some aspects. Under certain conditions, it can increase the sensitivity of chemotherapy in tumor cells by using autophagy to modulate the drug and change the level of autophagy in tumor cells, thereby improving the chemotherapy effect. In short, although there is still a certain distance from the actual clinical use of autophagy-regulating drugs, we expect that as the research progresses, the mechanism gradually clarifies, the experiment continues, and the experience gradually accumulates. In the future, some cancer patients use the ideal autophagy to modulate the drug.

Radiotherapy is an important component of the comprehensive treatment of malignant tumors, and it is the main treatment for tumors with surgical treatment and chemotherapy. Researchers have indicated that autophagy could enhance the sensitivity of radiotherapy. However, numerous studies have also shown that tumor resistance to radiation therapy is often associated with the upregulation of autophagy in a variety of tumor cell lines. DNA is the main target of ionizing radiation in radiation therapy. Direct and indirect damage of ionizing radiation can cause DNA damage such as DNA double-strand break (DSB), DNA single-strand break (SSB), and base damage. Autophagy has a great effect on the stability of DNA. It can decrease DNA damage by controlling the quality of mitochondria and regulating ROS levels. Autophagy can also affect the DDR process by recovering the key proteins needed in

the DDR process. Therefore, autophagy participates in DNA damage and post-injury repair caused by radiotherapy.

Therefore, autophagy plays a different role in tumorigenesis and tumor progression. The normal cells with autophagy are more likely to survive and in the late stage, autophagy leads to tumor cells survival in the harsh microenvironment and accumulates more mutations for further transformation. Of course, different tumor types and different tumor microenvironments may also lead to a different situation. Exploring autophagy as a new anticancer therapy is a promising research direction. Autophagy plays an important role in the suppression of tumor, at the same time, as a stress adaptive response, it can promote tumor cell survival and proliferation. In many tumor cells, autophagy prevents cell death caused by metabolic stress and anoxic microenvironment. Autophagy regulation may become a new antitumor therapy associated with traditional cytotoxic drugs or targeted drugs.

Chapter 20
Autophagy and Tumorigenesis

Wenting Liu, Yan Meng, Chen Zong, Shanshan Zhang, and Lixin Wei

Abstract Tumour cells are derived from normal cells that undergo numerous genetic and epigenetic mutations under various stresses. This process involves changes in many intrinsic cellular mechanisms and in the microenvironment. Understanding the process is important for preventing tumorigenesis and tumour recurrence. Numerous studies have shown that sputum autophagy not only plays an important role in tumorigenesis but also has a dual role in tumour suppression and cancer promotion. On the one hand, excessive autophagy can cause apoptosis and death, thereby inducing an autophagic death mechanism that leads to the death of drug-resistant tumour cells in malignant tumours. On the other hand, autophagy can mediate tumour escape and promote the survival of tumour cells. With the expansion of in-depth research, increasing evidence has shown that the specific role of autophagy in tumorigenesis may be related to the specific stage of tumour development and specific tumour type.

Keywords Tumorigenesis · Autophagy · Tumour cell death · Tumour cell survival

Tumorigenesis is a process by which normal cells undergo numerous genetic and epigenetic mutations under various stresses, gradually forming cancer cells and eventually developing into tumours. This process involves changes in many intrinsic cellular mechanisms and in the microenvironment. Understanding the process is important for preventing tumorigenesis and tumour recurrence. Numerous studies have shown that sputum autophagy not only plays an important role in this process but also has a dual role in tumour suppression and cancer promotion. On the one hand, excessive autophagy can cause apoptosis and death, thereby inducing an autophagic death mechanism that leads to the death of drug-resistant tumour cells in malignant tumours. On the other hand, autophagy can mediate tumour escape and promote the survival of tumour cells. With the expansion of in-depth research, increasing evidence has shown that the specific role of autophagy in tumorigenesis may be related to the specific stage of tumour development and specific tumour types.

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20.1 The Impact of Autophagy Dysfunction on Tumorigenesis

There are many research methods for discovering tumorigenic factors, but only detection in tissue samples from patients with cancer-related diseases and the study of the primary tumours in animal models come close to replicating the clinical reality. For the detection of the autophagy-related status of clinical samples, early detection of autophagy-related gene expression is the main method. With advancements in methodology, an increasing number of studies clearly show LC3 aggregation points through immunohistochemistry. This approach indicates the number of autophagosomes and is used as an indicator to judge the level of autophagy. On the other hand, with the development of genetic engineering technology, genetically engineered animal models, especially mouse models, have been widely used in cancer research and greatly promote our understanding of the factors affecting tumorigenesis. Here, we first review the advances in research on primary tumorigenesis in mouse models with impaired autophagy processes.

As early as 1963, Belgian cytologist and biochemist Christian de Duve proposed the concept of autophagy at the International Conference on Lysosomes in London, but it was not until 1997 that the Yoshinori Ohsumi laboratory cloned the first autophagy gene in yeast. *Apg1p* (the homologous gene in mammals is autophagy-related gene 1, *Atg1*) marks the official beginning of autophagy research. Direct evidence that autophagy can affect tumorigenesis was unveiled in a study of the key gene *beclin 1* in autophagy regulation at the Beth Levine Laboratory at the turn of the century. In 1998, Liang Xiaohuan et al. cloned the first mammalian autophagy gene, *beclin 1,* which is the homologue of *Atg6* in yeast. At this time, according to a review of the literature, the human chromosome 17q21, where *beclin 1* is located, has a single allele deletion in 40–75% of tumour tissues from patients with ovarian, breast or prostate cancer. The following year, the Liang Xiaohuan group also reported that endogenous *Beclin 1* protein is often expressed at low levels in human breast cancer cell lines and patient tumour tissues but is highly expressed in normal breast epithelial tissues. Moreover, the high expression of *beclin 1* can promote the activation of autophagy in the human breast cancer cell line MCF7 and inhibit its ability to form tumours in vitro and in vivo. These results suggest that *beclin 1* is likely to act as an autophagy-related gene that inhibits tumorigenesis in human breast cancer. Then, in 2003, Qu Xueping et al., also at the Beth Levine Laboratory, reported a study on mice with a *beclin 1* heterozygous gene deletion (*beclin* $1^{+/-}$). The results showed that the wild-type mice used in the study were in the advanced stage of spontaneously emergent lung cancer and experienced spontaneously occurring lymphoma, while *beclin* $1^{+/-}$ mice presented with the accelerated occurrence of the same type of lung cancer and lymphoma, spontaneously developed liver cancer and showed malignant progression of HBV-induced liver pre-cancerous lesions. In the same year, a study by Yue Zhenyu et al. confirmed that the loss of the heterozygous *beclin 1* gene significantly increased the probability of tumours spontaneously forming in mice, and in wild-type mice, only spontaneously formed lymphoma was

found, but *beclin 1*$^{+/-}$ mice showed spontaneously formed B cell lymphoma, lymphoblastic lymphoma, liver cancer, lung adenocarcinoma and other tumours. It is worth noting that in this mouse model of spontaneously formed tumours, the other wild-type allele of *beclin 1* remained undiscovered or had mutated. This finding is consistent with the same phenomenon observed in clinical tumour samples. These findings indicate that *beclin 1* is a tumour suppressor gene with an insufficient single allele, and it also suggests that although the autophagy level of cells in *beclin 1*$^{+/-}$ mice was significantly reduced, there remained a considerable degree of autophagy activation. The role of this low level of autophagy in the induction of tumorigenesis by deletion of the heterozygous *beclin 1* gene did not cause concern at first, but more studies of mouse models with autophagy-related gene deletion were reported and revealed that sex is an important factor, as recognized by more researchers.

In 2007, Yoshinori Takahashi et al. reported on the role of *Beclin 1* binding factor, *Bif-1*, deletion on tumorigenesis in mice. *Bif-1* was first discovered to activate Bax and Bak during apoptosis and had low expression in gastric cancer, and the homozygous form was deleted in mantle cell lymphomas. Yoshinori Takahashi et al. found that *Bif-1* is also a positive regulator of *Beclin 1* and binds to *Beclin 1* via UVRAG to activate autophagy. According to previous studies, UVRAG is often observed in single-allelic mutations in tumour tissues of patients with colon cancer, and UVRAG can inhibit the tumorigenic ability of human colon cancer cell lines in vivo. The action of *Bif-1* is somewhat different from that of *beclin 1* in that a *beclin 1* homozygous deletion in mice leads to embryonic lethality, while a *Bif-1* homozygous deletion in mice leads to normal development, and the phenotype has a significantly higher incidence in tissues of mutant mice compared to that of wild-type mice, except in spleen. Otherwise, no significant differences have been observed. However, the probability of tumours spontaneously forming in mice with a homozygous *Bif-1* deletion was significantly increased. The incidence of lymphoma and liver cancer was significantly higher in mutant than in wild-type mice; likewise, the incidence of sarcoma and oesophageal squamous cell carcinoma, which did not occur in wild-type mice, small cell lung cancer and duodenal adenocarcinoma was greater in mutants. In 2013, Yoshinori Takahashi et al. also reported that in Eμ-Myc mice, a single-allelic deletion of *Bif-1* accelerated proto-oncogene Myc-driven lymphoma by inhibiting mitochondrial autophagy (mitophagy). Unlike wild-type mice, *Bif-1*$^{-/-}$ in Eμ-Myc mice caused most foetuses to die, and the time to death was typically 9.5 days. However, of the Eμ-Myc mice that survived, no significant developmental defects were observed in *Bif-1* wild type or in *Bif-1*$^{+/-}$ or *Bif-1*$^{-/-}$ mutant mice. Further observations indicated that *Bif-1* deletion promoted lymphoma development in the Eμ-Myc mice, significantly shortening its survival, but between *Bif-1*$^{+/-}$ mice and *Bif-1*$^{-/-}$ mice, no significant difference was found in the occurrence of tumours or cell survival. These results indicate that *Bif-1* is also a tumour suppressor gene with an insufficient single allele.

In addition to *Beclin 1* and its binding proteins, other ATG proteins are of interest to researchers. Moreover, unlike *Beclin 1* and its binding proteins, ATG proteins have no other anticancer or anti-proliferative functions independent of autophagy, and research on them has more specifically demonstrated their role in autophagy.

In 2007, Guillermo Marino et al. reported on a study of mice with a homozygous *Atg4C* deletion. As one of the four homologous genes of *Atg4*, the homozygous deletion of *Atg4C* did not affect the expression of the other three homologues but did affect growth, development and reproduction under normal conditions and starvation conditions. There was no significant effect on autophagy activity (autophagosome aggregation tended to decrease when the diaphragm muscle tissue was starved but not to a statistically significant difference), and this activity did not increase the probability of spontaneous tumour formation. However, in the mouse model of fibrosarcoma induced by 3-methylcholanthrene (MCA), the homozygous deletion of *Atg4C* increased the likelihood of fibrosarcoma occurrence in mice and accelerated its formation, but the degree of tumour infiltration was not significantly affected, indicating that the loss of *Atg4C* can promote tumorigenesis without affecting the later development of the tumour. Studies on primary fibroblasts have shown that the loss of *Atg4C* inhibits autophagy activation during cell starvation. These results indicated that *Atg4C* has an important effect on the autophagy activity of cells under certain conditions in a specific tissue, which may be the reason its loss promotes carcinogen-induced fibrosarcoma.

Unlike *Atg4C*, most of the ATG genes are not homologous to each other because of their encoded proteins, which include *Atg3, Atg5, Atg7, Atg9,* and *Atg16L1*. The heterozygous deletion of these ATG genes was not observed to have a significant effect on tumorigenesis, but the homozygous deletion conferred a serious problem: neonatal lethality. Three to 12 h after birth, many tissues in the body had a significant upregulation in autophagy. The loss of the essential gene for autophagy caused the suckling mouse to die within a few days after birth. Although forced feeding could prolong its survival, such treatments could not reverse the course of its death. The study found that the concentration of amino acids and energy-related indicators in the mice with degraded organs were significantly decreased, which means that in the early stage after the foetus was removed from the placenta, energy production and protein synthesis in the body depended heavily on autophagy.

On the one hand, the solution to this problem relies on the development of animal model preparation techniques related to molecular biology, and on the other hand, the unknown factors of life also play a role. Akito Takamura of Noboru Mizushima Laboratories reported that the mosaic knockout of *Atg5* and hepatocyte-specific knockout of *Atg7* can both cause spontaneous liver cancer in mice. They originally wanted to use the Cre/loxp system for inducing system-specific knockouts of *Atg5*, but in a puzzling outcome, the hybrid mice generated from $Atg5^{flox/flox}$ mice and CAG-*Cre* mice all had the deletion in every organ. Further analysis with a partial knockout of *Atg5* revealed that 60–90% of cells in each tissue expressed the unaffected *Atg5* flox allele. The specific cause of this incomplete knockout is not known, but fortunately, in an animal model in which only some of the cells in the tissue had completely knocked out *Atg5*, the mice could survive for more than 19 months. These *Atg5*-mutant mice provided a good model for long-term observation of the fate of autophagy-deficient cells in the body. The Noboru Mizushima Laboratory, despite the unsuccessful construction of the expected experimental model, can continue to study autophagy with

rigor, which shows that fortune shines on those who are prepared. The final observations showed that after 6–9 months after birth, the *Atg5*$^{flox/flox}$; CAG-*Cre* mice presented with tumours on the liver, and at 19 months, the tumours had also spread throughout the liver, and all mice were observed to have a liver tumour. *Atg5*$^{flox/+}$; CAG-*Cre* mice and *Atg5*$^{flox/flox}$ mice did not have observable liver tumours until they were 19 months old. It is worth noting that in the *Atg5*$^{flox/flox}$; CAG-*Cre* mice, only the liver was observed to have undergone tumorigenesis, while in other tissues, despite having some cells with completely knocked out *Atg5*, such as lung, stomach, kidney and spleen, no tumours were found, for example, in the intestine, heart, brain and smooth muscle. To test whether this phenomenon is specific for *Atg5*, the researchers also constructed *Atg7*$^{flox/flox}$; Alb-*Cre* mice, in which they specifically knocked out the *Atg7* gene in the liver. Although some *Atg7*$^{flox/flox}$; Alb-*Cre* mice died of liver dysfunction 3 months after birth, most mice survived for more than a year and developed tumours on the liver. This tumorigenesis was due to the loss of autophagy rather than the specificity of an autophagy gene. Interestingly, a pathology analysis of the liver showed that the tumours in both models were benign. This is an important observation when analysing the role of autophagy in tumorigenesis.

These mouse models seem to indicate that autophagy plays a role in tumour suppression in tumorigenesis, but the complexity of life tells us that the reality is not so simple. In 2011, Wei Huijun et al. reported that knocking out the autophagy-related gene FIP200 can inhibit the occurrence of mouse breast cancer. FIP200 is an interaction protein of the focal adhesion kinase family. The molecular mass is 200 kDa. FIP200 participates in the formation of the ULK1-ATG13-FIP200-ATG101 complex, which plays an important role in the initial stage of autophagosome formation. In this study, Wei et al. used the MMTV-PyMT mouse model (FIP200^{Atg7} MMTV-*Cre* MMTV-PyMT mice), which had specifically knocked out FIP200 in mammary epithelial cells. MMTV-PyMT can mediate the activation of proto-oncogenes, including *Ras, Src* and *PI3K,* in mouse mammary epithelial cells and eventually leads to the occurrence of multiple breast cancers. These results showed that the targeted knockout of *FIP200* extended the average tumour occurrence time in mice from approximately 60 days to 85 days and that the survival time was prolonged from 100 days to approximately 140 days. The area of the breast occupied by the mammary epithelial tumours was also reduced by 60% at 10 weeks of age, and lung cancer metastases were reduced to approximately 1/10. The expression and function of the proto-oncogene *PyMT* in *FIP200*-mutant mice were not affected, while autophagy was significantly inhibited in tumour cells. The results are different from those obtained in previous models, suggesting that autophagy has a cancer-promoting effect.

In 2013, the Eileen White laboratory published two consecutive articles reporting the role of autophagy in the development of lung cancer induced by sustained activation of Ras or its activated downstream effector *Braf*. The experimental group led by Yanxiang Guo found that during the spontaneous formation of non-small cell lung cancer induced by *K-ras*$^{G12D-/+}$ in mice, despite the conditional knockout of *Atg7* (intranasal injection of *Cre*-containing adenovirus in *Atg7*$^{flox/flox}$ mice can cause *Atg7* knockout in some lung cells), the overall pathological condition did not

change, including tumour number, wet weight and tumour burden of the lung at the 10th week; in addition, the tumour growth rate was reduced, the airspace of the lung was no longer decreased and the tumour burden was reduced to approximately one-half that of the wild-type mice between the 14th and 16th weeks. It is worth noting that although hyperplasia developed into adenomas in the lungs of wild-type mice and *Atg7*-mutant mice from the 2nd week to the 6th week, adenomas began to transform into adenocarcinomas in wild-type mice between the 6th and 7th weeks, while the *Atg7*-mutant adenomas turned into benign oncocytomas. Later, between 18 and 42 weeks, many *Atg7*-mutant tumour cells decayed, causing the tumours to shrink.

The same laboratory group led by Anne M Strohecker used $Braf^{V600E}$ (a mutated protein in which the amino acid proline is exchanged for glutamate at position 600, resulting in sustained and *Ras*-independent activation of Bras) to promote the tumorigenesis of lung cancer in mice. Excessive hyperplasia was observed in the lungs of $Braf^{V600E}$ mice at 2–4 weeks, and scattered benign adenomas emerged at 6–8 weeks and then transformed into malignant adenocarcinomas only when the tumour suppressor gene *Ink4A/Arf* or *Trp53* was missing. $Braf^{CA-/+}$ mice ($Braf^{V600E}$ expression depends on the expression of *Cre*), $Braf^{CA-/+}\ Atg7^{flox-/+}$ mice and $Braf^{CA-/+}Atg7^{flox/flox}$ mice are also injected intranasally with *Cre*. The adenoviral sputum thus causes a small portion of the lung cells to express $Braf^{V600E}$ and form a tumour. The detection of $Braf^{CA-/+}Atg7^{flox/flox}$ mice also showed that the *Atg7* was only deleted in tumour tissue. Hyperplasia was found in the lungs of $Braf^{CA-/+}$ mice injected with virus at the 5th week, and the tumour burden was less than 10%. In the 7th week, a large number of scattered adenomas emerged and stably developed into papillary adenoma at 14 weeks. The average survival time was approximately 59 weeks. $Braf^{CA-/+}Atg7^{flox/flox}$ mice injected with virus had a lung tumour burden of up to 50% in the 5th week that had decreased to less than 30% by the 10th week. The average survival time was only approximately 85 weeks. The tumour occurrence increased in $Braf^{CA-/+}Atg7^{flox-/+}$ mice injected with virus. There was a small adenoma in the 5th week, but the average survival time was only approximately 187 weeks, and there was no difference compared with the $Braf^{CA-/+}$ mice. $Braf^{CA-/+}beclin\ 1^{+/+}$ mice and $Braf^{CA-/+}beclin\ 1^{-/+}$ mice were injected with virus. Similarly, for $Braf^{CA-/+}Atg7^{flox/flox}$ mice and $Braf^{CA-/+}Atg7^{flox-/+}$ mice, there was no significant difference in the mean survival time, which was 15 weeks and 18 weeks, respectively. At the same time, similar to $K\text{-}ras^{G12D/+}$ mice, the *Atg7*-mutant tumour cells were converted from adenomas to oncocytomas. These results indicated that autophagy deficiency can promote early tumorigenesis. However, loss of heterozygosity of the autophagy-associated genes had no significant effect on tumorigenesis. On the other hand, a complete autophagy deletion inhibited further tumour growth and malignant transformation.

In summary, an increasing number of studies have shown that autophagy has different effects at different stages of tumorigenesis. In the early stage of tumorigenesis, autophagy loss is mainly used as a driving force to promote the transformation of normal cells into benign tumour cells. In the late stage of tumorigenesis, autophagy

loss mainly affects the growth of benign tumour cells and inhibits malignant transformation. As the authors' laboratory reported in 2013, this phenomenon was also observed in a study of diethylnitrosamine-induced hepatocarcinogenesis in rats by the autophagy inhibitor chloroquine (CQ). Malignant tumours could be observed in rats with spontaneous tumour formation caused by the *beclin 1* heterozygous deletion. The reason for this finding might be related to low beclin-1 expression rather than complete gene deletion. The study of tumorigenesis induced by proto-oncogenes has also shown the inhibition of tumour growth and malignant transformation caused by autophagy deficiency. However, autophagy showed different behaviours in different tumour types. In the Ras-related pathway activation-induced lung cancer, autophagy inhibition led to tumorigenesis and tumour shrinkage at the late stage. This shrinkage was not observed in liver tumours induced by *Atg5* or *Atg7* deletion. The possibility that, as in the lung cancer situation, the liver tumours may be related to the Ras pathway has not been excluded. In addition, the deletion of autophagic alleles may lead to many changes in cells and tissues. Although there is a specific influence on tumorigenesis at a certain stage, either suppression or promotion, it does not mean that other factors with opposite effects do not exist during this stage.

20.2 Inhibition of Tumorigenesis by Autophagy and Its Mechanisms

20.2.1 Autophagy Inhibits the Inflammatory Response

An increasing number of studies have shown that the occurrence of tumours is closely related to chronic inflammation. Tumour development and the microenvironment on which tumours depend are composed of extracellular matrices, soluble molecules and tumour stromal cells. Numerous immune cells, such as T cells, myeloid suppressor cells, and macrophages, are the main components of the tumour stroma. Along with the infiltration of inflammatory cells, a large number of cytokines and chemokines play a non-negligible role in the process of tumour development. An increasing number of studies have shown that inflammatory cells and molecules are involved in dynamic processes that reflect the evolution of the tumour microenvironment, and the final outcome is the accumulation of a large number of inflammatory cells and factors that promote tumorigenesis. The interactions between tumorigenesis and local or systemic inflammatory responses are highly complex and diverse (Zhong et al. 2016).

In recent years, studies have shown that autophagy affects tumorigenesis by regulating inflammation. Since Beth Levine's laboratory group found that autophagy plays an important role in tumorigenesis, more people are investigating the mechanisms involved. In 2006, Kurt Degenhardt et al. of the Eileen White Laboratory first reported the inhibition of inflammation by autophagy. Inflammation, especially chronic inflammation, is considered to be an important characteristic of tumour

formation. The inflammatory microenvironment can provide pro-survival factors, growth-promoting factors, pro-angiogenic factors, etc., thereby promoting the acquisition of other key features in tumour formation. Unlike apoptosis, which does not easily induce an inflammatory response, necrosis releases numerous pro-inflammatory factors into the surrounding tissue environment, induces macrophage infiltration and promotes the production of various pro-inflammatory factors, making it an important inflammation-inducing factor. Although the death of tumour cells, especially those unable to undergo apoptosis, is increased, which can inhibit tumour growth, the increase in inflammation due to increased necrosis may lead to the opposite result. Degenhardt et al. found that the loss of autophagy reduced the survival of immortalized renal epithelial cells that lost the ability to undergo apoptotic under metabolic stress, resulting in increased necrosis, increased inflammatory response and ultimately accelerated tumorigenesis. It has also been seen in other studies that the phenomenon of indirect inhibition of the inflammatory response by autophagy is mostly related to inhibition of necrosis. In the process of spontaneous non-small-cell lung cancer (NSCLC) formation induced by *K-rasG12*$^{D/+}$ in mice, the necrosis caused by the knockout of *Atg7* increased, which also led to a marked increase in the expression of various inflammatory-related genes in the tumour-bearing lung tissue and even a violent inflammatory response. It was the main cause of death of the mice studied.

In addition to indirectly inhibiting the inflammatory response, autophagy can also directly regulate the formation of inflammatory factors. Members of the inflammatory factor IL-1 family (including IL-1α, IL-1β and IL-18) have a significant pro-inflammatory effect. IL-1α, IL-1β and IL-18 promote the production of IFN-γ and IL-17. IL-1α and IL-1β can recruit a variety of bone marrow cells, including neutrophils, to the site of inflammation and can induce cyclooxygenase 2, phospholipase A2 and inducible nitric oxide synthase (iNOS) generation. IL-1β has also been reported to promote the secretion of IL-1α and IL-23. In 2008, Tatsuya Saitoh et al. reported that the loss of *Atg16L1* in mice resulted in the production of more IL-1β and IL-18 by macrophages stimulated by endotoxin lipopolysaccharide (LPS). Increased IL-1β is associated with activation of caspase-1 by Atg16L1 deletion. Cadwell et al. found that Atg16L1 deficiency inhibited the production of antimicrobial peptides, leading to excessive accumulation of intestinal microbes, stimulating the secretion of IL-23 and IL-17, and ultimately leading to the development and progression of colorectal cancer. Since then, more research reports have shown that autophagy exerts an important influence on the production and secretion of the IL-1 family. On the one hand, in macrophages and dendritic cells, autophagy can induce inflammasome activation from inflammatory stimuli by reducing reactive oxygen species (ROS), mitochondrial DNA, protein aggregates, and others. Thus, Toll-like receptor-mediated secretion of IL-1α, IL-1β and IL-18 is reduced, and ROS itself can also act as a signalling molecule to promote the expression of TNFα and IL-6. On the other hand, in macrophages, autophagy directly degrades the inflammasomes NLRP3 and AIM2, as well as the IL-1β precursor. Both NLRP3, an intracellular sensor, and AIM2, a DNA sensor, promote caspase-1 activation, and caspase-1 activates the precursors of IL-1β and IL-18, enabling them to mature and be secreted. Guo Wenjie

et al. reported that andrographolide also inhibits the activation of NLRP3 by promoting mitochondrial autophagy in macrophages, thereby inhibiting the occurrence of colitis-related tumours.

Autophagy not only regulates the formation of inflammatory factors but also affects the differentiation and function of inflammatory cells. In the process of inflammatory reactions, whether inflammatory cells can differentiate and mature normally is the point of bifurcation that regulates the secretion of inflammatory factors and affects the inflammatory response. In the complex inflammatory reaction process, there are many kinds of inflammatory cells, and each has its own role. For different inflammatory cells, the effect of autophagy is also very different (Fridman et al. 2017; Zhong et al. 2016).

In recent years, a large number of studies have found that autophagy can affect the inflammatory process by regulating inflammatory cells. Dendritic cells (DCs) constitute an important component of the inflammatory process by presenting antigens to $CD4^+$ and $CD8^+$ T lymphocytes, thereby initiating immune surveillance and inflammatory responses. However, autophagy is involved in the regulation of TIMD4 inhibition of DC antigen presentation by degrading antigens (Baghdadi et al. 2013). Hubbard-Lucey et al. found that the deletion of *Atg16l* promoted the activation of DCs. Moreover, autophagy restricts DCs from presenting glycolipid antigens to NK cells, inhibiting the occurrence of inflammatory reactions (Keller, et al. 2017). As an important component of the inflammatory response, macrophages are not only responsible for removing necrotic fragments of damaged tissues and cells but also responsible for secreting a large number of inflammatory factors that play an important role in the inflammatory reaction process. Degenhardt et al. found that autophagy plays a role in the inhibition of the inflammatory response by macrophages. The intervention of macrophage autophagy can significantly increase the production of the inflammatory factors IL-1β and IL-18 (Degenhardt et al. 2006). Deletion of *Atg16L1* promotes the accumulation of TRIF in macrophages and the secretion of its downstream inflammatory factors IFN-γ and IL-1β (Saitoh et al. 2008). Deletion of *Pik3c3* in $CD4^+$ T cells interferes with the homeostasis and function of T cells and specifically knocks out *Atg5* or *Atg7* in regulatory T cells, leading to the expression of FOXP3, which inhibits the differentiation and maturation of T_{reg} cells, causing a serious inflammatory immune response (Marcel and Sarin 2016; Wei et al. 2016). Grivennikov et al. further confirmed that autophagy limits the polarization of Th17 cells by inhibiting the production of IL-1β and that intervention autophagy significantly enhances the activation of Th17 cells and the development of colon cancer (Grivennikov et al. 2012).

20.2.2 Autophagy Participates in Maintaining Genomic Stability

The so-called development of things is the result of interactions between internal and external factors. The effect of autophagy on tumorigenesis is important not only for affecting external causes of inflammation but also for inducing changes in the intrinsic mechanisms of cells. In 2007, the Eileen White laboratory published two consecutive articles. Robin Mathew et al. and Vassiliki Karantza-Wadsworth et al. reported that loss of autophagy promotes DNA damage in immortalized renal epithelial cells and immortalized mammary epithelial cells under metabolic stress. Gene amplification, aneuploidy formation and genomic instability ultimately promote tumorigenesis. On the one hand, metabolic stress leads to the misfolding and aggregation of proteins, which interferes with DNA replication and repair; on the other hand, it increases the burden of mitochondria and leads to an increase in ROS production. ROS not only participate in the inflammatory reactions in macrophages but also activate responses in other types of cells. They also damage proteins, DNA and organelles such as mitochondria, which in turn leads to greater oxidative stress and metabolic stress. ROS alone or the misfolding and accumulation of many proteins can activate autophagy, but in the absence of autophagy clearance, both lead to further accumulation of protein aggregates or damaged organelles, which may be the reason for the ultimate increase in genomic instability. At the same time, the increased sensitivity of cells to metabolic stress caused by the loss of autophagy can also lead to a decrease in cell survival, which may be related to the accumulation of the excess cellular content described above. In tissues, the cycle of 'cell death and compensatory hyperplasia' caused by persistent damage, the "injury repair" cycle and its accompanying inflammatory response, and even tissue repair activate tissue stem cells to participate in repair in vivo. Cell damage is considered to have an important position in the early stage of tumorigenesis. In other words, regardless of the type of cell, the specific environment, or the fate of an autophagy-deficient cell under metabolic stress—whether life or death—the cycle promotes tumorigenesis; that is, the life and death possibilities of a metabolically challenged cell are opposite sides of the same plane of existence that are complementary and equally important.

This dual nature of autophagy may also explain why the mosaic-type knockout of *Atg5* mice in the experiments of Akito Takamura et al. ultimately led only to the spontaneous formation of liver tumours. Although the accumulation of p62 is also observed in other tissues, the metabolic pressure from liver function is the greatest, which leads to increased misfolded protein aggregation in the hepatocytes with autophagy loss and increased aggregation of damaged organelles, thus leading to more accumulated p62. However, liver tissue has a proliferative capacity far beyond that of other tissues. On the one hand, the autophagy-deficient cells that accumulate too many undesirable contents die. On the other hand, the accumulation of fewer autophagy-deficient cells causes DNA damage that, stimulated by dead cells, leads to compensatory proliferation such that the cycle is repeated under metabolic pressure. Other tissues under less metabolic stress, despite some autophagy-deficient cells

accumulating p62, have fewer contents that cause adverse effects in autophagy-deficient cells, and therefore, it is rare that cell-injury pressure causes a cycle of life and death. In the end, tumours are less likely to occur.

Xie Rui et al. reported that upregulated microtubule-associated proteins 1S (MAP1S) activates autophagy during DEN-induced hepatocarcinogenesis in rats, thereby clearing damaged mitochondria and p62. Labelled protein aggregates reduce DNA damage and maintain cell genomic stability, thereby inhibiting tumorigenesis, while autophagy activation also attenuates DNA damage and genomic instability within tumour tissues. However, whether this attenuated DNA damage makes it possible to suppress the malignant transformation of the tumour is debatable. DNA damage is also a double-edged sword for the occurrence and development of tumours. It is also a quantitative problem. Appropriate DNA damage within an acceptable range of cells will cause the cells to accumulate mutations. Under environmental screening, the number and variety of predatory cells survive and proliferate, and eventually, their slow accumulation will cause qualitative changes that are manifested by the genomic instability of tumour cells and their transformation into malignant cells. However, if the tumour cells are faced with rapid quantitative accumulation, that is, excessive DNA damage, despite having advantages in tolerating DNA damage and DNA repair, then they still die, and there is no malignant transformation of cells. Autophagy, on the other hand, hinders DNA damage, enabling cells to face relatively mild DNA damage. This impediment to death by DNA damage may also be the cause of tumorigenesis in benign tumours in mice with complete autophagy function.

Although these studies suggest that autophagy plays an important role in protecting the genomic stability of the cell, the exact mechanism by which autophagy deletion leads to genomic instability remains unclear. It is worth noting that tumorigenesis is a multistep, complex process, but mature somatic cells usually have very limited proliferative capacity unless the first stable mutation is a strong proliferative-related gene; otherwise, it is difficult to carry enough multiple-effect mutations to create an algebraic expression of proliferation. Moreover, even if a key proliferative gene in the cell is overexpressed, if there is no corresponding gene to assist, then it may accelerate proliferation but not satisfy the proliferation requirement due to the synthesis of mechanistic macromolecules that initiate apoptosis or ageing. In addition, there is a corresponding genomic stability maintenance mechanism in the cell that detects and repairs DNA damage, making the likelihood of genome mutation in successive generations of cells extremely low. On the other hand, the accumulation of cell damage often leads to apoptosis, ageing and even necrosis, which makes it difficult for normal cells to develop into tumour cells. Therefore, an important question prompts an answer to how autophagy-deficient cells under the influence of injury factors generate cells proliferation algebra for survival and eventually accumulate a large number of DNA mutations that lead to genomic instability.

Some studies in recent years have shown that the effect of autophagy loss on stem cells may hold the key to answering these questions. Early tumorigenesis, if not driven by proto-oncogene activation due to genetic factors, is often a slow and sustained process of damage repair. Loss of autophagy promotes cell death, which

accelerates this cycle of life and death. The continuous damage repair cycle stimulates corresponding stem cells to support the repair process. Loss of autophagy also enhances stem cell damage. Stem cells carrying DNA mutations and their daughter cells are continuously screened under internal and external pressure. Cells carrying mutations that promote survival live in such an environment and further accumulate non-lethal DNA damage, ultimately leading to genomic instability. Moreover, autophagy has a special role in stem cells. Monika Mortensen et al. reported that knocking out *Atg7* in the mouse haematopoietic system results in loss of the normal function of the haematopoietic stem cells and causes severe lethal myeloid proliferation. *Atg7*-mutant haematopoietic stem progenitors have increased mitochondrial accumulation and ROS, as well as accelerated proliferation and increased DNA damage. Despite the increased proportion of haematopoietic stem cells, this population of cells lost the ability to differentiate downstream, meaning that lymphoid progenitor cells and myeloid progenitor cells were missing (Mortensen et al. 2011). The research team led by Guan Junlin reported that knockout of *FIP200* in neural stem cells also affected its differentiation function and was closely related to the increase in ROS (Wang et al. 2013). The proliferation of dying progenitor cells and the decreased ability to differentiate may be early events in tumorigenesis. These studies suggest that accelerating the repair cycle of stem cell involvement may play an important role in genomic instability caused by the loss of autophagy (Fig. 20.1).

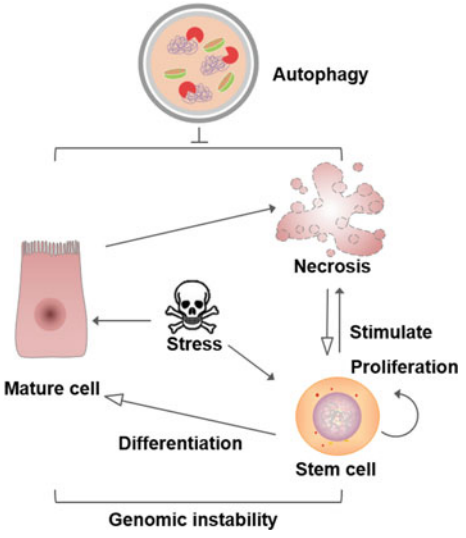

Fig. 20.1 Autophagy may inhibit the occurrence of genomic instability by slowing the damage repair cycle in which stem cells are involved

20.2.3 Autophagy Promotes Cell Senescence

Cellular ageing is also an important way for the body to prevent cancer of normal cells. The proliferation of proto-oncogenes such as *Ras, Myc* and *Braf* can only be promoted by other proto-oncogenes or mutations of tumour suppressor genes such as *TP53* and *Rb*, which may be insufficient for normal cells to meet the requirement for rapid cell proliferation. Relevant support is required. A single individual proto-oncogene mutation will lead to cell cycle arrest, cell senescence, apoptosis or other means of death. Studies have shown that autophagy is involved in the process of oncogene-induced senescence (OIS). Andrew R. J. Young et al., in a report in 2009, explored the senescence process of the human embryonic lung fibroblast IMR90 induced by H-RasV12. The results of the study showed that autophagy was significantly elevated during this OIS process, and this increase was associated with the inhibition of mTORC1 and mTORC2 activities of mTOR, but a role for mTOR-independent pathways was not ruled out. Further studies have shown that activation of ULK1 by inhibition of mTORC1 and decreased activity of FoxO3a by inhibition of mTORC2 play important roles in OIS. After the inhibition of autophagy, H-RasV12-induced senescence of IMR90 was significantly inhibited, but the inhibition of autophagy did not reverse the ageing process of senescent cells, suggesting that autophagy is likely to play a major role in the initial establishment of senescence. It was subsequently found that the production of the senescence-associated secretory factors IL-6 and IL-8, which play key roles in this OIS, was also significantly hindered by inhibition of autophagy. However, other means such as knocking out the *Rb* gene to inhibit cell senescence cannot inhibit the production of IL-6 and IL-8, suggesting that the effect of autophagy on IL-6 and IL-8 expression is direct; that is, it is not conferred indirectly through the suppression of senescence. Although it is not yet certain how autophagy affects IL-6 and IL-8 expression during OIS, these effects have shown autophagy to be important in promoting ageing.

Liu He et al. also found a similar phenomenon in the occurrence of melanoma. They tested samples from patients and found that the expression of *Atg5* and the level of autophagy in the melanoma tumours were significantly lower than they were in the benign melanin tumours and that *Atg5* was highly expressed in patients with melanoma with low expression of *Atg5*. Progression-Free Survival (PFS) was significantly prolonged. Both clinical samples and cell line assays have shown that downregulation of *Atg5* expression in melanoma is not associated with gene mutations but that the promoter of the *Atg5* gene is methylated. Overexpression of *Atg5* in melanoma cells significantly inhibits cell proliferation and induces cell senescence. Downregulation of *Atg5* expression also promotes the proliferation of human melanocytes expressing $Braf^{V600E}$ or $H\text{-}ras^{G12V}$ and inhibits the induction of senescence by these two proto-oncogenes. These results suggest that *Atg5* and autophagy are activated during melanoma development and play a role in inhibiting tumorigenesis by inducing cellular senescence.

20.2.4 Autophagy Inhibits the Accumulation of P62/SQSTM1

Associated with connexins and having many protein binding regions, p62 is degraded in an autophagy-dependent manner. Therefore, the accumulation of p62 is considered to be a classic indicator of the inhibition of the autophagic process. The accumulation of p62 is more than just an indicator; an increasing number of studies have shown that it has a direct role in promoting tumorigenesis.

Angeles Duran et al. first found that mice with completely knocked out p62 were resistant to *Ras*-induced lung cancer. Since then, more studies have shown that p62 deletion inhibits the *Ras*-driven conversion of normal cells to tumour cells and reduces the anchorage-independent growth of human hepatoma cells while also inhibiting spontaneous liver cancer growth in *Atg7* knockout mice. Among these lines of research, the role played by p62 in RAS-driven tumour cells is currently being studied. It is generally believed that p62 activates nuclear regulatory factor 2 (NRF2) and NF-κB to stimulate angiogenesis and pro-inflammatory responses, respectively, thereby promoting tumour growth. An increase in autophagy leads to the degradation of p62, which in turn attenuates angiogenesis and inflammatory responses. In recent years, an increasing number of studies have suggested that P62 itself is carcinogenic. Umemura and other studies have found that overexpression of P62 alone can induce hepatocellular carcinoma (Moscat et al. 2016).

NRF2 acts to promote angiogenesis and enhance cell survival by regulating a range of genes. Under normal conditions, it is ubiquitinated by the E3 ubiquitin ligase Kelch-like ECH-associated protein 1 (KEAP1), which degrades it. The long-term accumulation of p62 by over-activated NRF2 promotes the transcriptional activation of its downstream target gene Sqstm1, and the expression of Sqstm1 further promotes the accumulation of p62 to induce tumorigenesis in a long-term continuous process (Moscat et al. 2016). In non-small cell lung cancer, abnormal activation of the NRF2 pathway due to a keap1 mutation is considered an important mechanism for promoting tumour cell survival. In cells with deficient autophagy, the accumulated p62 directly binds to KEAP1, thereby interfering with NRF2 ubiquitination and degradation, leading to abnormal activation of NRF2-mediated-related pathways, which likely includes the autophagy process and is, therefore, an important mechanism involved in the failure of cells to survive.

20.3 Promoting the Effect of Autophagy on Tumour Formation and Understanding Its Mechanisms

Although numerous experiments have shown that inhibition of autophagy enhances tumorigenesis, a mouse model of a deleted autophagy gene suggests that inhibition of autophagy also inhibits tumorigenesis. This study showed that autophagy promotes

the tolerance of tumour cells to stress and increases metabolic adaptability. A certain basal level of autophagy is important for the survival and development of tumour cells.

20.3.1 Autophagy Promotes Cell Survival by Resisting Internal and External Stress

During the process of further growth and transformation of original tumour cells, the rapid growth phase is an important stage. At this stage, tumour cells proliferate to generate a large number, and the new blood vessels are limited, resulting in a partial lack of local oxygen and nutrient supply. The contradiction between the rapid proliferation of tumour cells and the relative inferiority of the external environment leads to a dramatic increase in damage factors such as oxidative and metabolic stresses. It creates a momentous obstacle to tumour cell survival and proliferation. The activation of autophagy plays an important role in overcoming this hurdle.

Nutritional deficiency is an important factor in inhibited tumour growth. It is also a powerful inducer of autophagy. The main function of autophagy is to eliminate damaged cell structures, ageing organelles and biological macromolecules that are no longer needed (such as misfolded proteins) to provide raw materials for cell reconstitution, regeneration and repair and to recycle and reuse intracellular substances. Inhibition of the mTOR pathway plays an important role in the induction of autophagy. mTOR is a sensor of the metabolic state. Amino acids and growth factors can promote the activity of mTORC1. mTORC1 is sensitive to rapamycin and can induce autophagy under certain conditions, such as those stemming from a lack of nutrition, stress, and growth factor signal reduction. Activated autophagy can recycle intracellular substances by degrading damaged proteins, organelles and other biomacromolecules to meet the necessary biological and energy synthesis needs in the cell under conditions of insufficient nutrient and energy supplies. In addition, the lower intracellular amino acid level also relieves its inhibition of the RAS/RAF1/ERK pathway, thereby activating autophagy. Glucose is the main source of ATP production, while AMPK is the intracellular energy sensor. Elevation of the AMP/ATP ratio can significantly activate AMP. Therefore, low concentrations of glucose activate autophagy in a manner that is partially dependent on AMPK. In addition, the ability of tumour cells to take up glucose is significantly higher than that of normal cells. Autophagy also plays a role in this process, but the specific mechanism is not clear.

Angiogenesis refers to the growth of capillary blood vessels derived from capillaries. Tumour angiogenesis is an extremely complex process that includes the microvascular growth induced by tumour cells and the establishment of blood circulation in tumours. Tumour angiogenesis is an important condition for tumour development. Blocking the formation of tumour neovascularization and cutting off the supply of

tumour nutrients can achieve "tumour starvation therapy" for the purpose of inhibiting and treating tumours. Min et al. found that acetylation of cyclophilin A is an important regulator of hypoxia-induced autophagy and angiogenesis in lung tumours. Under hypoxic conditions, miR-195 targeting GABA-receptor associated protein-like 1 (*GABARAPL1* gene) promotes autophagy and angiogenesis of endothelial progenitor cells (hepc cells), thereby regulating cell proliferation. Vascular endothelial growth factor (VEGF) is a key regulator of tumour angiogenesis. VEGF can bind to the vascular endothelial growth factor receptor and promote vascular endothelial cell proliferation and neovascularization in colonic malignant tumours. Enhancing autophagy activity to increase the permeability of vascular endothelial cells and promoting neovascular invasion leads to tumour invasion and metastasis (Sousa et al. 2016). Ruan et al. found that the P13K/AKT/mTOCR signalling pathway acts as a 'tumour angiogenesis' regulatory centre and regulates VEGF expression under hypoxic conditions (Ruan and Kazlauskas 2012). Although mTOCR activity is inhibited in hypoxic conditions, cells can release hypoxia-inducible factor-1α (HIF-1α) via the mTOCR signalling pathway. Activation of mTOCR can increase the expression of HIF-1α in tumour tissues to promote angiogenesis in normoxic environments. A study in a rat-based model showed that anti-angiogenic therapy combined with chemotherapy can improve the overall survival rate of patients with ovarian cancer and that anti-angiogenic therapy is accompanied by a 'rebound effect' led by hypoxia-induced autophagy, increasing tumour angiogenesis, accelerating tumour growth and promoting vascular leakage; in contrast, inhibition of autophagy can improve the prognosis of patients with cancer.

Hypoxia is an important feature of tumour microenvironment. Hypoxia can increase the expression of HIF-1, while HIF-1 can upregulate the expressions of BNIP3 and BNIP3L. BNIP3 and BNIP3L can activate autophagy by linking with beclin-1. ROS can cause damage to DNA, proteins and organelles, and this damage and metabolic stress can lead to autophagy activation. Even the activation of autophagy by starvation conditions is related to ROS. The stress of hunger can lead to the activation of JNK. Thus, phosphorylation and decoupling of Bcl-2 from beclin-1 induces autophagy, and ROS are also involved in the process. Autophagy activation prevents subsequent damage and inhibits apoptosis by removing these damage-related factors. Anti-angiogenic therapy combined with chemotherapy can improve the overall survival rate of patients with ovarian cancer.

Chaperone-mediated autophagy (CMA) may also play an important role in combating oxidative damage by affecting PKM2 levels. CMA is upregulated in a variety of tumours, especially in gliomas. M2-pyruvate kinase isoform (PKM2) is the rate-limiting enzyme of glycolysis, which is upregulated in various tumours. Previous studies have shown that the PKM2-mediated conversion rate of phosphoenolpyruvate to pyruvate is lower than that of PKM1. The accumulation of glycolytic intermediates provides raw materials for the synthesis of other biomacromolecules and promotes tumour cell proliferation. Lewis Cantley et al. found that maintaining PKM2 at a low level can promote the metabolism of sugar metabolites in the antioxidant pathways of tumour cells, which confers resistance to oxidative stress. CMA plays an important role in maintaining a low level of PKM2 by selectively degrading PKM2.

In the absence of nutrients, tumour cells upregulate autophagy activity by activating the mutated proto-oncogene *K-Ras* or *H-Ras* to maintain their own oxidative metabolic activity. The proto-oncogene *p53* can inhibit autophagy, but a harsh microenvironment, similar to that of tumour cells, causes degradation of p53, thereby activating autophagy and promoting tumour cell growth. Excessive production of multiple proteins driven by overexpression of proto-oncogenes such as *MYC* can cause great stress on the endoplasmic reticulum, which acts as a multifunctional organelle for protein synthesis and transmembrane transport, post-translational modification, glycosylation, cholesterol and phospholipid synthesis, and intracellular Ca^{2+} homeostasis, which are closely related processes. Protein glycosylation modification is particularly important for protein quality control. Misfolded proteins are retained in the lumen of the endoplasmic reticulum and cause endoplasmic reticulum stress. Once the accumulation of excess or misfolded proteins exceeds a threshold level, mitochondria-dependent and independent apoptotic pathways are activated. Autophagy participates in the clearance of excess or misfolded proteins to promote cell survival.

Lipid metabolism changes significantly in tumours. Fat and cholesterol synthesis are significantly increased in prostate cancer. Fatty acid oxidation is also an important source of energy for prostate cancer cells. However, excessive lipid accumulation is also unfavourable for tumour cell survival. Lipophagy is an important means of degrading fat granules in adipose tissue. Autophagy also regulates lipid metabolism in hepatocytes. The complete knockout of *Atg5* leads to hydrolysis of triacylglycerol in hepatocytes. However, it is unclear whether this mechanism is established in tumour cell lipid metabolism. At the same time, it has been reported that mitochondrial autophagy can also affect lipid metabolism. In *KRAS*-driven, *Tp53*-mutant non-small cell lung cancer cells, knockout of *Atg7* can cause intracellular lipid accumulation. This is based on the increase in fatty acid oxidation caused by dysfunctional mitochondria, which inhibits the growth of tumour cells and transforms them into eosinophils, which are not malignant.

Inhibition of the immune response is one of the other ways that autophagy promotes tumour cell survival. Inhibition of autophagy by genetic means not only inhibits tumour growth in tumour-bearing mice but also enhances immune surveillance and enhances CD8+ T cell-mediated cytotoxicity. The tumour eventually kills. For example, in the *ATG4*B-negative dominant pancreatic ductal adenocarcinoma (PDAC) mouse model, autophagy was limited, and significant inhibition of tumour growth was observed. When a normal autophagy cell was injected into nude mice, the tumour 'returned', *ATG4*BCA+ (autophagy-deficient) cells did not. A recent PDAC study showed that inhibition of autophagy can cause recruitment and infiltration of antitumour T cells and CD68+ macrophages. *FIP200* knockdown in the MMTV-PyMT mouse model of induced breast cancer resulted in increased secretion of multiple chemokines by the tumour cells, including CXCL9, CXCL10, CXCL11, CCL5 and CCL8, and led to greater CD8 infiltration, which is capable of triggering IFN production and CD4 cell infiltration to promote tumour cell death and inhibit tumour growth. However, autophagy inhibits the secretion of many cytokines in tumour

cells. Which of these cytokines plays a major role, is this phenomenon autophagy- or FIP200-specific, and is it unique to breast cancer? These questions require further investigation.

20.3.2 Autophagy Maintains Cellular Metabolism

20.3.2.1 Autophagy and Mitochondrial Oxidative Metabolism

To adapt during oxygen and nutrient deficiency, tumour cells change their metabolic mode from aerobic metabolism to hypoxia metabolism and improve their efficiency in the utilization of limited nutrients in various ways. Autophagy plays an important role in this process. Similar to aerobic conditions, tumour cells tend to undergo glycolysis, and some tumour cells also show considerable dependence on autophagy under nutrient-rich conditions. In addition to the factors that require tumour autophagy to clear misfolded proteins and damaged organelles, the metabolism of tumour cells may also be one of the reasons for their dependence on autophagy.

Pancreatic ductal adenocarcinoma (PDAC) is the most common pathological type of pancreatic cancer. K-ras mutations can be detected in more than 90% of PDACs. RAS activation can significantly increase the level of autophagy in cells. Compared with normal pancreatic cells, in primary PDACs, the level of basal autophagy is increased significantly. Inhibition of autophagy not only significantly inhibited the proliferation of PDAC cells in vitro but also inhibited the growth of PDAC-implanted tumours in mice. Similar phenomena have been observed in immortalized or tumour cells transfected with RAS. Activation of KRAS significantly accelerates the intracellular metabolic rate. Cells require faster energy production and macromolecular synthesis to meet the need for increased cell proliferation. Autophagy is an important condition for maintaining the metabolic mode of RAS-transfected cells. The loss of autophagy causes mitochondrial oxidation, ATP production and macromolecular synthesis in such cells. The level of tricarboxylic acid (TCA) is significantly decreased, which significantly inhibits the viability and proliferation of PDACs. The mechanism of autophagy promoting tumorigenesis contributes to the survival of cells under many pressures; it also serves as an important support for the special metabolism of tumour cells. However, Mohamed Elgendy et al. showed that RAS induced Noxa and *beclin 1* expression, which promoted autophagic cell death and inhibited the ability to clone the affected cells. Why does RAS cause autophagy-dependent death, but the survival of tumour cells overexpressing RAS depend on autophagy? In addition to the different cell lines used in each experiment, the different outcomes are explained by whether RAS is acutely overexpressed and overexpressed stably over time. Acutely induced RAS overexpression activates autophagy, and it has no corresponding gene mutations, which leads to the activation of intracellular cell cycle arrest or cell senescence signalling pathways, leading to cell death. However, cells with stably overexpressed RAS survived through the screening. They overcame the pressure of proto-oncogenes and were challenged by more metabolic

pressures due to the demand for rapid proliferation. Autophagy activation has played a role in alleviating metabolic stress. The difference between these two states is consistent with the anticancer and cancer-promoting effects of autophagy in the early and late stages of tumorigenesis, respectively.

Researchers from the United States have demonstrated that conditional knockout of the autophagy gene *Atg5* (*Atg5*-KO) extends the survival of $KRAS^{G12V}$-driven tumours in mice by 38%. Despite rapid onset, the *Atg5*-KO tumours spread slowly during advanced tumorigenesis. *Atg5*-KO tumour cells showed decreased mitochondrial function and increased mitochondrial rupture. Despite the compensatory overexpression of asparagine synthetase, the metabolite profile showed a deficiency in the non-essential amino acid asparagine. Inhibition of autophagy or asparagine synthetase reduced $KRAS^{G12V}$-driven tumour cell proliferation, migration and invasion, which could be rescued by asparagine supplementation or knockout of MFF (mitochondrial fission factor) (Lin et al. 2018).

In addition, the dependence of tumour cell metabolism on autophagy may also be related to the effect of proto-oncogenes. The PI3K-related pathway has many functions, such as promoting cell growth, proliferation, and metabolism. It is abnormally activated in various tumours, and it also activates mTOR, which leads to the inhibition of autophagy. Chen Nan et al. reported that in a 3D growth model of mammary epithelial cells that present constant activation of type I PI3K, autophagy mainly inhibited rather than promoted proliferation. Overexpression of p62 can significantly promote the proliferation of such cells. This aspect of proliferation may be related to RAS activation of autophagy. Type I PI3K is involved in the inhibition of autophagy. On the other hand, the proliferation may also be related to the role of p62. It may also be related to the nutrient microenvironment in the tumour growth model. However, the specific situation requires more research with an activated proto-oncogene model.

20.3.2.2 Autophagy and Glucose Metabolism

Glycolysis is common in tumour cells. Even in the case of sufficient oxygen, tumour cells are more prone to aerobic glycolysis than aerobic oxidation. Tumour cells prefer to undergo aerobic glycolysis because it helps them gain access to the accumulated metabolic intermediates they need for macromolecular synthesis. Mitochondrial autophagy and CMA play important roles in the transformation of tumour cells from undergoing aerobic oxidation to preferring aerobic glycolysis.

The number of mitochondria plays a role in the transformation of aerobic metabolism. Many studies have reported that BRAF-overexpressing melanoma cells have a reduced rate of mitochondrial synthesis, which promotes their switch from aerobic oxidation to aerobic glycolysis, and increased mitochondrial autophagy can have a similar effect. RCAN1-1L expression was induced during oxidative stress, which can lead to the permeability of mitochondrial transition pores and reduce ATP levels. This expression is inhibited by mTOR in the AMPK pathway, which further increases mitochondrial autophagy and promotes cell conversion to aerobic glycolysis

Reports by Maria Kon et al. showed that inhibition of CMA inhibits the occurrence of glycolysis in tumour growth. Selective degradation of PKM2 by CMA may be a key step in its promotion of aerobic glycolysis. PKM2 is the rate-limiting enzyme for glycolysis. Degradation of PKM2 by CMA can further upregulate the levels of various glycolysis intermediates such as glucose-6, glucose-1,6-diphosphate and ATP. It has been reported that the specific knockout of *PKM2* can significantly promote the deletion of *Brca-1* during breast cancer formation; antioxidation and increased levels of aerobic glycolysis as a result of PKM2 knockout might be involved in the process.

20.3.2.3 Autophagy and Amino Acids

Amino acids are essential for the growth and proliferation of tumour cells. The raw amino acid material produced by autophagy-degraded proteins can be used to supply biosynthetic pathways that meet the needs of the rapid cell division and proliferation of tumour cells. Glutamine is the most abundant amino acid in cells and plays an important role in the development of tumours. A study of pancreatic cancer has shown that inhibition of autophagy in pancreatic astrocytes reduces the exposure of alanine in tumour cells, thereby leading to the suppression of tumour cell metabolism and growth (Sousa et al. 2016). As the rate of glycolysis accelerates, tumour cells become more dependent on glutamine decomposition to supplement the raw materials in the TCA cycle and maintain ATP production. In pancreatic cancer, intermediates of glutamine metabolism are involved in TCA. In addition, when glutamine is deficient, the expression of glutamine synthetase is upregulated. The PI3K-PKB¯FOXO signaling pathway is thought to play a role in this process. Conditional activation of FOXO3 can significantly increase glutamate production. At the same time, it inhibits mTOR through glutamine synthetase and finally activates autophagy. This FOXO3-mediated glutaminase-induced autophagy plays an important role in the survival of colon cancer cells. Loss of autophagy in wild-type embryonic fibroblasts leads to a decrease in intracellular glutamine levels and a similar metabolic change due to glutamine deficiency, suggesting that autophagy plays an important role in maintaining the intracellular content level of glutamine content; however, loss of glutamine does not increase the level of autophagy, and the level of *Atg5* mRNA is also reduced. It has been reported that glutamine deficiency activates the intracellular total amino acid control pathway. It upregulates the expression of amino acid transporters, thereby increasing the uptake of amino acids, which in turn leads to the activation of mTOR and subsequent inhibition of autophagy (Chen et al. 2014). Therefore, the effect of the concentration of glutamine on autophagy in tumour cells and how autophagy participates in the maintenance of specific amino acid levels when tumour cells are starved need further study.

20.4 New Theory of Tumorigenesis: Adaptive Selection of Tumour Cells in the Inflammation Microenvironment

Traditional research suggests that tumorigenesis is a process in which normal cells in tissues undergo many genetic and epigenetic variations under the stimulation of various cancer-inducing factors, gradually forming cancer cells and eventually developing into tumours. However, more and more studies have confirmed that the occurrence of tumours is closely related to inflammatory damage, and most normal cells die in the inflammatory injury microenvironment, thus losing the time and opportunity to form tumours. The source and mechanisms of tumour cells are important scientific issues in understanding tumorigenesis. In recent years, research has gradually formed a new view that tumorigenesis is due to the continuous apoptosis and necrosis of normal mature cells in the inflammatory microenvironment, and the abnormal differentiation of immature precursor cells into tumour cells to adapt to the harsh inflammation microenvironment. Taking hepatocarcinoma (HCC) as an example, chronic inflammatory injury leads to normal hepatocyte apoptosis, and hepatic progenitor cells (HPCs) are activated. The proliferating HPCs normally differentiate into hepatocytes or biliary cells to repair the liver. However, under the action of the harsh microenvironment, HPCs are abnormally differentiated into hepatoma-initiating cells, leading to the occurrence of liver cancer. Studies have shown that the activation of HPCs in adjacent tissues of liver cancer is closely related to tumour recurrence. The more the number of HPCs activated, the higher the probability of tumour recurrence. HBV infection is an important risk factor for the occurrence and recurrence of HCC. HBV infection induces the inflammation microenvironment in the liver, which leads to hepatocyte necrosis. HPCs are activated and abnormally differentiate into tumour cells that do not express HBV receptors, so they are not easily attacked by HBV and escape death, eventually lead to the formation of tumour (Jing et al. 2015). Based on the results of the relevant studies, we propose a new theory of tumorigenesis: adaptive selection of tumour cells in a harsh inflammatory microenvironment (Fig. 20.2). In addition, the results of the recent application of the tyrosine metabolism-deficient mouse liver cancer model further support this theory. Normal hepatocytes express Fah, a key enzyme in the tyrosine metabolic pathway. In the absence of Fah, the metabolites of tyrosine cannot be decomposed and accumulate in the liver, thereby inducing hepatocyte death. In the microenvironment of liver injury induced by Fah deletion, HPCs are activated and differentiate into tumour cells by metabolic reprogramming, while hydroxyphenylpyruvate dioxygenase (HPD) gene is deleted in tumour cells, resulting in no metabolites to escape the damage, and ultimately lead to the formation of liver cancer. Lipopolysaccharide is an important pro-inflammatory factor in the tumour microenvironment, which not only induces the secretion of downstream inflammatory factors IL-6 and TNF-α. It can also promote the activation of HPCs and inhibit its normal differentiation, and further induce the abnormal differentiation of HPCs involved in the formation of liver fibrosis and HCC (Liu et al. 2019; Pan et al. 2017). TNF-α has also been reported

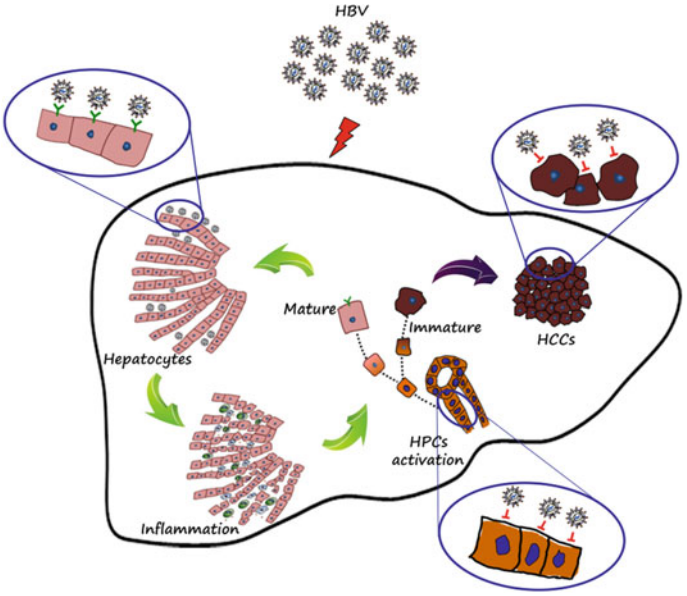

Fig. 20.2 HBV infection leads to massive necrosis of normal liver cells. HPCs are activated in this harsh inflammatory microenvironment, but because the surface does not express HBV receptors, it can escape the necrosis induced by HBV infection, and adapt to the inflammatory microenvironment, eventually forming a tumour with abnormal differentiation. Differentiated tumour cells can also escape their infection due to the lack of HBV receptors (Oncotarget, 2015, 6(40): 42952–62)

to be closely associated with activation and abnormal differentiation of HPCs (Jing et al. 2018). The above results indicate that the harsh inflammatory microenvironment can lead to normal cell damage and induce tissue stem cell repair response, while stem cells further differentiate into tumour cells to adapt to the harsh inflammatory microenvironment and ultimately lead to tumorigenesis. This theory also explains well for the plasticity, heterogeneity, and stemness of tumour cells.

20.5 Conclusions

Numerous studies have shown that autophagy has always played a role in protecting cells throughout the tumour stage, but it plays a different role in tumour suppression and cancer promotion in the early and late stages. This finding is mainly related to the mechanism protected. Autophagy in the early stage of tumorigenesis is important for inhibiting inflammation, maintaining cell genomic stability, promoting damaged cell senescence and inhibiting the accumulation of P62, thus protecting normal cells from instability and inhibiting their transformation into tumour cells. In the late stage of tumorigenesis, the initial tumour cells have formed and are growing and

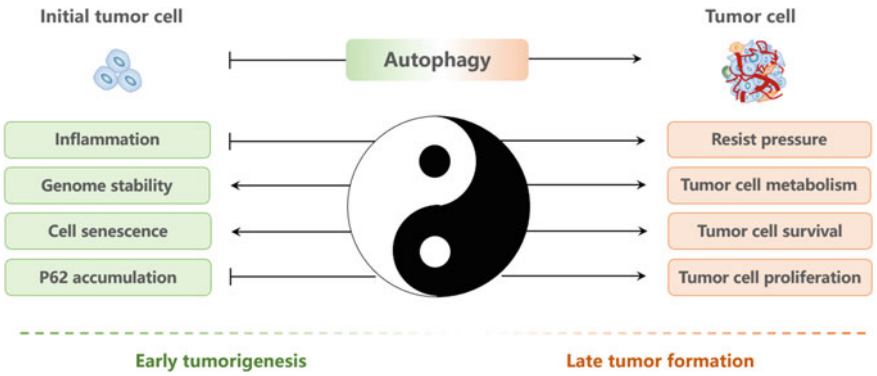

Fig. 20.3 Autophagy plays a role in tumour suppression and in cancer promotion in the early and late stages of tumorigenesis, respectively

transforming despite the relatively infertile environment. In this stage, autophagy mainly promotes tumour cell survival and proliferation by helping it resist internal and external stresses and inducing the vigorous metabolism characteristic of tumour cells (Fig. 20.3).

Therefore, there may be two useful screenings related to autophagy in the process of tumour survival. Normal cells demonstrating normal autophagy are more likely to transform into tumour cells, and in the late stage, autophagy leads to tumour cell survival in the harsh microenvironment, where it accumulates more mutations for further transformation. Of course, different tumour types and different tumour microenvironments lead to different late-stage situations.

Although a large number of autophagy-related studies have greatly enhanced our understanding of the role of autophagy in tumorigenesis, there are still many aspects worthy of further exploration. Autophagy is associated with the stemness maintenance of cancer stem cells in certain tumours. Cancer stem cells have a higher level of autophagy or higher autophagic tolerance. The mechanism that determines the induction of the different mechanisms of cancer stem cells and non-stem cells needs to be made clear. Autophagy is related to the proliferation and differentiation of normal stem cells; are only ROS and mitochondrial autophagy involved? Is autophagy also directly involved in the degradation of factors related to stemness and differentiation? What is the role of autophagy in the malignant transformation of normal stem cells? The tumour microenvironment plays an important role in the development of tumours. Autophagy has also been found to be involved in many of the cells, such as macrophages, mesenchymal cells, and tumour-associated fibroblasts, among others, in the microenvironment. Does autophagy play an important role in tumour-related mechanisms? Answers to these questions are important for clarification.

With the continuous development of autophagy research, autophagy is increasingly included in cancer therapies. Autophagy plays a key role in promoting and inhibiting therapies. However, to better evaluate the effects of each therapy, it is necessary to have a more comprehensive understanding of the role of autophagy at

various stages and in aspects of tumorigenesis. This understanding will also lead to new and more effective therapies for cancer treatment.

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Chapter 21
Autophagy and Tumour Stem Cells

Xue Yang, Fei Ye, Yingying Jing, and Lixin Wei

Abstract Autophagy is critical for the survival and stemness maintenance of cancer stem cells (CSCs) and is an enhancer of CSC tumorigenesis. At the same time, autophagy contributes to conditions optimal for facilitating the invasion and metastasis of CSCs. Moreover, autophagy induces the dormant state of CSCs to help them resist the cytotoxic effects of chemotherapy and radiotherapy, thereby improving the likelihood of their survival. The combination of autophagy inhibitors with specific drugs targeting specific CSC subpopulations is expected to act specifically on CSCs and produce fewer toxic side effects on normal tissues. This in-depth study is very timely and important for further identifying the potential role of autophagy in different states of CSCs and places a particular emphasis on exploring molecular mechanisms in the regulation of autophagy via advanced techniques based on molecular biology.

Keywords Autophagy · Tumour stem cells · Stemness maintenance · Survival

There is a rare type of cancer cell in tumour tissue that has the potential for self-renewal, proliferation and differentiation, and it plays an important role in the processes of tumour occurrence, development, recurrence and metastasis. Due to their many properties that are similar to those of stem cells, they are called cancer stem cells (CSCs). First discovered in the haematopoietic system, cancer stem cells have been identified in a variety of tumours through the detection of various markers such as CD44, CD24, Epcam and CD133 (Clevers 2011; Meng et al. 2012; Wang et al. 2015). They divide asymmetrically, separating into cancer stem cells with an identical nature and into non-tumorigenic cancer cells that make up the bulk of tumours. In the process of tumour development, a tumour microenvironment characterized by hypoxia, reduced pH and nutrient deficiency is formed. However, in such an environment, which is harsh for normal cells, cancer stem cells can grow well. What is the mechanism that helps cancer stem cells cope with the harsh environment in tumour tissues? Autophagy is believed to enable the body to survive under severe living conditions by

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'degrading itself to provide materials and energy and remove harmful substances'. At present, a large number of studies have confirmed that autophagy plays an important role in cell differentiation, development and adaptation to environmental stress and contributes to the occurrence of a variety of diseases. However, a large number of studies have also found that during the emergence and development of tumours, autophagy plays a dual role. In normal tissues, autophagy-mediated injury relief can effectively inhibit tumorigenesis, while in tissues with tumours, the macromolecular recycling induced by autophagy can alleviate only the energy demands of cancer stem cells to maintain their survival. In this case, inhibition of autophagy is beneficial for antitumour therapy. However, both the inhibition and activation of autophagy inhibit tumour growth by removing cancer stem cells. We all know that the most important feature of stem cells is their ability to self-renew and differentiate. Currently, increasing evidence has suggested that autophagy is involved in the resting, self-renewal and differentiation of stem cells during physical and other kinds of stress conditions. Is the function of autophagy in CSC self-renewal and differentiation similar to that of other stem cells? Or do cancer stem cells have other special regulatory functions not present in other stem cells? Recent studies have suggested that in addition to its role in normal embryonic development and adult stem cells, autophagy also plays an important role in the origin, maintenance and migration of cancer stem cells. The role of autophagy in maintaining the survival and function of cancer stem cells is described below.

21.1 Autophagy Is Involved in the Maintenance of the Stemness of Cancer Stem Cells

Tumour stem cells comprise a small subgroup of cells within the tumour that are closely related to the growth, drug resistance, recurrence and metastasis of the tumour. Tumour stem cells have the potential for self-renewal and differentiation into a variety of somatic cells and can maintain their undifferentiated state, thus leading to the heterogeneity of tumour tissues. Transplantation of a small number of tumour stem cells resulted in tumour formation in immunodeficient mice (Sharif et al. 2017). Numerous reports have shown that many factors are related to the maintenance of the stemness of tumour stem cells, and among them, the POU domain transcription factor (POU5F1) plays an important role during embryonic development and pluripotency maintenance of tumour stem cells (Ng and Surani 2011). In addition, cytokines secreted by tumour cells, such as interleukin-6 (IL-6) and transforming growth factor-β (TGF-β), can stimulate the transformation of some tumour cells into tumour stem cells. In addition, it has been reported that the activation level of autophagy increases in a variety of tumour stem cells, such as breast cancer stem cells, pancreatic cancer stem cells and liver cancer stem cells. The high infiltration and rapid growth of tumour cells lead to insufficient blood supply inside the tumour, which results in hypoxia. Simulation of the intermittent hypoxic environment in vitro could reprogramme pancreatic cancer

cells such that they obtain a stem-like phenotype and promote the transformation of pancreatic cancer cells into $CD133^+$ tumour stem cells, which are mainly dependent on autophagy activation induced by hypoxia-inducible factor-1 (HIF-1α). In a breast cancer study, researchers found that both genetic and pharmacological inhibition of cell autophagy activity can make breast cancer stem cells more inclined to show an epithelioid phenotype while inhibiting their ability to show mesenchymal phenotypes, such as the $CD44^+$ $CD24^{low}$ immune phenotype, which is often considered a marker of breast cancer stem cells. The same phenomenon was found in ovarian cancer. In ovarian cancer stem cells, whether the autophagy level is downregulated by CQ or by interference from ATG5, the pellet-forming ability of ovarian cancer stem cells and the expression of dry genes are inhibited, and FOXA2 is involved in this downregulation. Regarding the process whereby cirrhosis of the liver leads to the development of liver cancer, researchers found that autophagy can drive $Axin2^+$ cells to become $Axin2^+$ $CD90^+$ cells, that CD90 is the marker of liver cancer stem cells and that high expression of the other stem cell markers OCT4 and SOX2 promotes colony formation in vitro and tumour formation in nude mice, in which HGF signalling pathways are involved (Peng et al. 2017). The results described above suggested that autophagy can promote the acquisition of tumour stem cells and the maintenance of their stemness (Peng et al. 2017). With ongoing research, different opinions have emerged. A report in 2017 made a unique point; that is, in a study of teratoma cells, researchers used inhibited or promoted autophagy levels in different ways and obtained the same result: stemness markers of cancer stem cells were reduced, and differentiation markers were increased; hence, they noted that maintaining the balance of the foundational autophagy level is vital to maintaining the stemness of cancer stem cells (Sharif et al. 2017).

21.2 The Role of Autophagy in the Adaptation of Tumour Stem Cells to Harsh Environments

In the process of tumour development, the tumour also forms a tumour microenvironment characterized by tissue hypoxia, decreased pH, nutritional deficiency and tumour angiogenesis. The ‘seed and soil’ theory is used to explain these phenomena: tumour stem cells are seeds, while the tumour microenvironment is the soil on which the tumour stem cells live. However, the soil is not warm and fertile but relatively barren. Local hypoxia and a relative lack of nutrients caused by insufficient blood supply are common in solid tumours and are important characteristics of the tumour microenvironment. At the start of tumorigenesis and metastasis, tumour stem cells must face and overcome this unfavourable tumour microenvironment. However, autophagy is actually a survival mechanism cells use in response to adverse environments, and hypoxia and hyponutrition are typical autophagy-inducing factors. In the environment of nutrient deficiency, cells use autophagy and lysosomes to degrade the damaged organelles and macromolecules and thus maintain the balance of protein

metabolism, stabilize the intracellular environment and provide the minimum energy required for cell survival.

Studies have confirmed that hypoxia can induce autophagy in tumour cells by hypoxia-induced factor-1α (HIF-1α), while hypoxia-induced autophagy can promote tumour cell survival and even promote the proliferation of tumour stem cells. Studies have found that $CD133^+$ hepatocellular carcinoma stem cells have higher levels of basal autophagy and ischaemia deficiency-induced autophagy than $CD133^-$ cells in vitro, whereas inhibition of autophagy reduced their viability, suggesting that cancer stem cells with higher levels of autophagy may respond more rapidly to the anoxic microenvironment. In addition to hypoxia, an important feature of the tumour microenvironment is ischaemia, which refers to a lack of nutrients. In addition to escaping biophysical limitations, starving cancer cells must be able to utilize renewable resources by activating catabolic processes, i.e. recycling intracellular components to maintain the balance of intracellular metabolism and cell viability. The function and activity of cancer stem cells depend on their ability to ensure adequate bioenergy supplementation in a timely manner. An increasing number of studies have found a close relationship between cancer stem cells and autophagy and have also suggested that the protein metabolism function of autophagy has a protective effect when tumour stem cells face starvation. The expression level of autophagy-related genes was significantly greater in cells with globular proteins that could be isolated from ductal carcinoma lesions. Treatment with the autophagy inhibitor chloroquine induced apoptosis in genetically abnormal spheroid cells, which were eventually cleared. Allograft tumours cannot be formed by reducing the expression of autophagy-related genes. The above evidence suggested that autophagy is critical for the survival of tumour stem cell-like precursor cells in pre-malignant lesions. Of course, in addition to helping cancer stem cells cope with harsh conditions in the tumour microenvironment, autophagy can also help the survival of cancer stem cells that are treated with chemotherapeutic drugs. Curcumin is considered as a chemotherapeutic drug that can induce tumour cell apoptosis through a variety of mechanisms. Researchers have found that while curcumin induces the apoptosis of colon cancer stem cells, autophagy is activated, thereby promoting colon cancer stem cells survival and enhancing their tolerance to the chemotherapeutic drug. However, unfortunately, there is still no literature that explains how autophagy can increase the tolerance of cancer stem cells to stress environments while maintaining their function and even promoting their expansion.

21.3 Autophagy and Tumour Stem Cell Apoptosis

Apoptosis refers to the self-ordered death pattern of cells controlled by genes to maintain homeostasis. Apoptosis is a fundamental biological phenomenon of cells that plays a necessary role in the removal of unwanted or abnormal cells by multicellular organisms. Tumour cells, especially cancer stem cells, have a prominent feature in which their proliferative capacity is abnormal and apoptosis is inhibited.

Therefore, the treatment of tumours should not be limited to killing tumour cells and inhibiting tumour cell division, and studying the induction of the apoptosis of cancer stem cells will be an important direction for antitumour research for a long time. In recent years, it has been found that in tumour stem cells, autophagy can not only promote cell survival but also regulate the apoptosis of cancer stem cells under certain conditions. At present, the study of the regulation of autophagy on apoptosis has created different perspectives. In the process of using a higher concentration of a plant-derived chemotherapeutic drug, rottlerin, to induce apoptosis in human prostate cancer stem cells, researchers have also found autophagy in tumour stem cells (cytoplasmic vacuoles and autophagosomes were observed under an electron microscope, and autophagy-related gene expression levels were significantly increased), and treatment of cells with the autophagy inhibitor 3-methyladenine could inhibit the apoptosis of cancer stem cells. This research suggested that the activation of autophagy in cancer stem cells may induce apoptosis. On the other hand, studies have shown that autophagy can inhibit apoptosis of cancer stem cells. In a study of breast cancer, the use of the autophagy inhibitor chloroquine could induce apoptosis in both breast cancer cells and breast cancer stem cells. The combination of chloroquine and chemotherapeutic drugs could enhance chemosensitivity. Quinacrine, another autophagy inhibitor, could also induce the apoptosis of breast cancer stem cells while inhibiting lysosomal acidification (Han et al. 2018). The mechanism by which autophagy regulates apoptosis is not clear but has been studied in tumour cells. In a leukaemia study, the researchers found that the autophagy inhibitor spautin-1 could promote cell apoptosis while inhibiting autophagy. This pro-apoptotic phenomenon was associated with activation of the key effector molecule GSK3β downstream of PI3K/Akt and downregulation of the apoptosis-related proteins Mcl-1 and Bcl-2. Therefore, the inhibitor has potential application in the treatment of tumours (Liu et al. 2011; Shao et al. 2014). A related study on adipose tissue-derived stem cells indicated that inhibition of autophagy promotes glucose-induced apoptosis and that ROS/JNK signalling pathways are critical in the induction of autophagy (Li et al. 2018).

21.4 Bidirectional Regulation of Autophagy on Glucose Catabolism of Tumour Stem Cells

In normal mammalian cells, glycolysis is inhibited under aerobic conditions. However, German biochemist Warburg found that under oxygen-rich conditions, malignant cells are also active in glycolysis. This aerobic glycolysis is a metabolic characteristic called the Warburg effect. It is characterized by a high glucose uptake rate, active glycolysis, and high lactic acid content of metabolites. Glycolysis not only produces ATP efficiently but also avoids the generation of unwanted endogenous reactive oxygen species (ROS). In addition, glycolysis can support the defence efficacy of

antioxidants by inducing the production of NADPH, which is required for the formation of key antioxidant enzymes, and reducing glutathione. Studies have suggested that autophagy may have a certain promoting effect on the level of glycolysis during stemness acquisition and malignant transformation of tumour stem cells. Loss of autophagy reduces glucose uptake, resulting in insufficient levels of glycolytic activity in cancer stem cells, which ultimately reduces the ability of mammalian stem cells to proliferate. Autophagy levels were found to be continuously activated in Kras-mutated pancreatic epithelial tumours and advanced pancreatic ductal carcinomas but not in normal pancreatic epithelium or early pancreatic epithelial tumours. Inhibition of autophagy can slow the proliferation and tumorigenesis of pancreatic cancer cells by reducing the oxidative phosphorylation of cancer stem cells. This finding suggests that autophagy promotes the mitochondrial oxidative phosphorylation activity of tumour cells. Urinary epithelial tumour stem cells showed a high level of autophagy, and the expression of glycolytic-related genes was also high. Inhibition of autophagy reduced the expression of glycolytic genes in cancer stem cells. The results suggested that autophagy promoted cell survival by buffering the bioenergy requirements necessary to meet the high levels of glycolytic reactions in cancer stem cells (Ojha et al. 2016). On the other hand, studies have suggested that autophagy may also act as an antagonist of the Warburg effect in specific cases. A study on acute leukaemia indicated that rapamycin induced autophagy but inhibited glycolysis and promoted cell proliferation (Watson et al. 2015).

21.5 Autophagy Required for the Migration and Tumorigenicity of Tumour Stem Cells

It is well known that tumour metastasis is one of the important biological characteristics of malignant tumours and one of the main factors for poor prognosis in patients with malignant tumours. Although 90% of tumour cells can ‘escape’ early from the primary tumour and eventually reach the pre-metastasis stage, fewer than 2% of these cells can form micro-metastatic deposits, and only 0.02% or fewer of these cells eventually develop into metastatic tumours, which means that tumour cells with certain intrinsic properties can maintain self-renewal and proliferation in the microenvironment throughout the complete metastatic process. Tumour stem cells showing aggressive invasiveness and tumorigenicity are the best candidates for being such cells. In recent years, studies have found that autophagy is essential for the survival of cancer stem cells. One study found that SDCBP/MDA-9/Syntenin signalling-mediated protective autophagy plays an important role in anoikis-resistant glioma stem cells (Talukdar et al. 2018). Autophagy inhibitors not only increase the death of chronic myeloid leukaemia tumour stem cells but also combine with tyrosine kinase inhibitors to significantly suppress their stem cell-like properties and functions. On the other hand, the epithelial to mesenchymal transition (EMT) plays an important role in tumour invasion and metastasis. An increasing number of studies

have found that cancer stem cells have an EMT phenotype that can confer migratory and invasive properties to tumour cells, giving them the characteristics of stem cells. Autophagy also plays an important role in the maintenance of the interstitial properties of tumour stem cells, including their migratory and invasive characteristics. Autophagy can promote tumour invasion and metastasis through the renewal of local adhesion molecules and upregulation of metastatic cytokine secretion. Studies have shown that autophagy promotes proto-oncogene RAS-mediated invasion by secreting the pro-metastatic cytokine IL-6. Autophagy can also decompose focal adhesions to promote the dissociation of locally adhered molecules and the migration of metastatic tumour cells through the combination of the autophagy molecule LC3 and paxillin regulated by the proto-oncogene SRC (Sharifi et al. 2016). Moreover, the autophagy-related genes for BECN1 and ATG5 gene silencing can inhibit autophagy in highly metastatic hepatoma cells and significantly suppress pulmonary metastasis of HCC in mice by impairing the anoikis resistance and colonization of the HCC cells. Autophagy inhibition may promote EMT through the ROS/HO-1 pathway in ovarian cancer cells (Zhao et al. 2016). In addition, the autophagy-related molecules DRAM1 and p62 regulate the migration and invasion of malignant glioma tumour stem cells. Increasing data from clinical studies have confirmed that autophagy corresponds to targeted cancer therapy. The autophagy core protein ATG4B is a potential biomarker and therapeutic target for chronic myeloid leukaemia $CD34^+$ haematopoietic stem/progenitor cells (Rothe et al. 2014). Autophagy-related factors are highly expressed in highly invasive tumour cells. The Thr300Ala variant of ATG16L1 is associated with a decreased risk of brain metastasis in patients with non-small-cell lung cancer (Li et al. 2017b). These studies have demonstrated that autophagy maintains the stemness and survival of tumour cells and enhances their invasive and migratory abilities. These studies also have provided new therapeutic strategies and targets for clinical intervention.

In addition to high metastatic ability, cancer stem cells also show high levels of tumorigenicity and demonstrate other biological characteristics. A very small number of cancer stem cells can be cultured in vitro to generate tumour cell colonies and can also form tumours when injected into experimental animals; however, in these cases, a large number of tumour cells are needed to form tumours. The highly tumorigenic characteristic of cancer stem cells has always been an important obstacle for the cure rate of tumours. Therefore, understanding the mechanism for inducing high tumorigenicity in cancer stem cells is conducive to improving the prognosis of patients. In recent years, studies have found that autophagy is required not only for the invasion and migration capacity of cancer stem cells but also for its role in tumorigenicity. Researchers isolated a subset of cells with stem cell characteristics from the bladder cancer cell lines T24 and UM-UC-3, and high levels of autophagy were observed in these side populations without any autophagy-inducing factors. Compared with other cells, the mortality of the side population cell groups was significantly increased after the inhibition of autophagy, and the colony formation ability was reduced for those with weak tumorigenicity. Similar studies have demonstrated that hypoxia-inducible factor-1 α-induced autophagy played an important role in the conversion of non-stem pancreatic cancer cells into $CD133^+$ pancreatic cancer stem-like cells (Zhu et al.

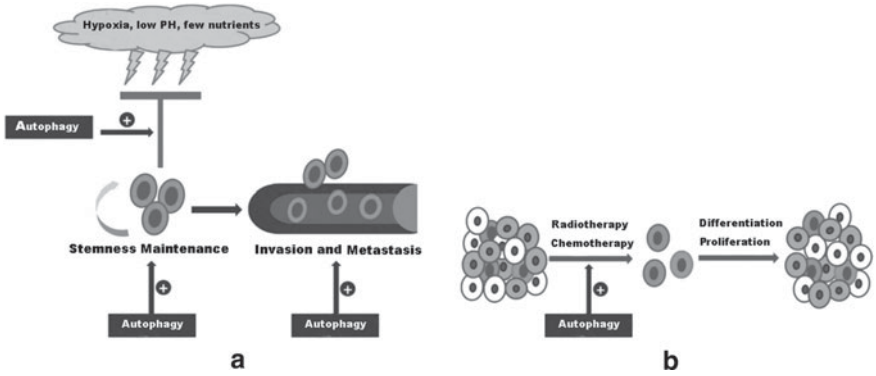

Fig. 21.1 Autophagy and tumour stem cells. **a** Autophagy participates in the maintenance and regulation of the biological characteristics of cancer stem cells. **b** Autophagy promotes the chemoradiation resistance of cancer stem cells

2013). Beclin 1 and autophagy were required for the tumorigenesis of breast cancer stem cells. In the pathologic microenvironment of cirrhosis, autophagy-dependent generation of $Axin2^{+}$ cancer stem-like cells promoted the development of hepatocellular carcinoma (Li et al. 2017a). Recent observations have suggested that autophagy promotes tumour-like stem cell niche occupancy (Zhao et al. 2018). Autophagy can also maintain stemness by preventing senescence (Garcia-Prat et al. 2016). In this regard, there are also different research perspectives, and studies have shown that autophagy suppresses the self-renewal and tumorigenicity of glioma-initiating cells by promoting the degradation of Notch1 (Tao et al. 2018). The studies described above confirm that autophagy contributes to the survival and stemness maintenance of cancer stem cells and enhances the tumorigenicity of cancer stem cells. At the same time, autophagy also contributes to conditions suitable for the invasion and metastasis of cancer stem cells that have high metastaticity. Traditional cancer treatments combined with autophagy inhibitors enhance the therapeutic antitumour effect (Fig. 21.1).

21.6 Autophagy and Cancer Stem Cells with Resistance to Chemotherapy and Radiotherapy

In addition to surgical treatment, radiotherapy and chemotherapy have become two of the most important treatments for cancer. Radiotherapy and chemotherapy can act quickly on tumour lesions and reduce tumour burden. However, the largest challenge encountered in current chemoradiotherapy treatments is the resistance of tumour cells to chemotherapy and radiotherapy, which leads to tumour recurrence. A large number of recent studies have shown that tumour resistance to radiotherapy and chemotherapy is mainly achieved by the cancer stem cells, while radiotherapy and

chemotherapy are used for selectively destroying dividing cells. However, current studies have found that autophagy is critical for the dormancy and quiescence of haematopoietic stem cells and muscle stem cells. The stem cells can switch from dormancy into metabolically active cells and can be prevented from irreversible ageing. Therefore, autophagy confers a dormancy phenotype in tumour stem cells to help them escape the cytotoxic effects of chemotherapeutic treatment and continue to survive. How to reduce the chemosensitivity of cancer stem cells has become a hot topic in the study of tumour therapy. At the same time, research on the mechanism of action for cancer stem cell resistance to radiotherapy and chemotherapy has become more urgent.

On the one hand, a large body of evidence indicates that autophagy plays an important role in the radiotherapy resistance of cancer stem cells. In gliomas, the $CD133^+$ cell population increased after radiation, and the survival rate of $CD133^+$ cells was significantly higher than that of $CD133^-$ cells. Further studies found that radiation can induce autophagy in glioma stem cells, and the level of autophagy in $CD133^+$ cells was significantly higher than that of $CD133^-$ cells, and the sensitivity of $CD133^+$ cells to radiation was enhanced after inhibition of autophagy. The survival rate of tumour stem cells and the colony-forming ability in vitro decreased significantly after irradiation, and while $CD133^-$ was inhibited after autophagy, the sensitivity of these cells to radiation was only slightly enhanced, suggesting that cancer stem cells play a leading role in resistance to radiotherapy. This study suggested that the induction of autophagy contributes to the response of glioma stem cells to the toxic effects of radiation and increases their rate of survival. In addition, it was found that the radiotherapy protocol induced the upregulation of autophagy-related genes in tumour cells, upregulated the expression of beclin 1, atg3, atg4b, atg4c, atg5 and atg12 and induced the aggregation of phagosomes. Inhibition of autophagy-related genes enhanced the sensitivity of tumour cells to radiotherapy, but researchers also found that the basal clonogenicity of untreated resistant cells may even be enhanced by the inhibition of autophagy. The results suggested that different tumour types and different tumour states, phenotypic characteristics of tumour cells and different individual inflammatory microenvironments in patients with tumours need to be considered for tumour therapy when autophagy is inhibited to optimize the options for cancer treatment.

Autophagy has also been found to be involved in the chemoresistance characteristics of cancer stem cells. Chronic myelogenous leukaemia (CML) stem cells were the first cancer stem cells to be isolated. Imatinib mesylate (IM) is the standard therapeutic drug for treating CML, but CML stem cells instinctively produce resistance to IM and accelerate the progression of CML. IM-induced autophagy can counteract tumour cell death, and autophagy inhibitors help restore the sensitivity of CML stem cells to IM treatment. In addition, the toxic effects of fluorouracil on colon cancer $CD133^+$ cells were significantly enhanced through autophagy inhibition. Another study found that the chemotherapy drug 5-FU and cisplatin induced the death of oesophageal cancer cells. It was found that chemotherapy-sensitive oesophageal cancer cell lines showed increased apoptosis, while chemotherapy-resistant oesophageal cancer cell lines showed increased autophagy. The study found that autophagy in

response to chemotherapy is the mechanism that promotes cell recovery and enhances chemotherapy resistance. The results suggested that selective inhibition of autophagy regulators could increase the effect of chemotherapy. In addition, the study found that the expression of the autophagic molecule LC3 in pancreatic cancer tumour tissues was closely related to the expression of the tumour stem cell markers aldehyde dehydrogenase 1 (ALDH1), CD133 and CD44. In pancreatic cancer cell lines, higher LC3-II expression was observed in the sphere-forming cells. The elevated expression levels of LC3-II suggested elevated levels of autophagy, and the researchers found that the chemotherapy drug gemcitabine combined with autophagy inhibitors had a significant effect on pancreatic cancer (Yang et al. 2015). In addition, studies have found that the level of autophagy in ovarian cancer stem cells was significantly higher than it was in non-stem cells, and the combination of the chemotherapy drug carboplatin and autophagy inhibitors improved the therapeutic effect on ovarian cancer (Pagotto et al. 2017). The increased autophagy level of tumour stem cells in the face of chemoradiotherapy is also a spontaneous protective behaviour of cells in harsh environments. Autophagy for cancer stem cells can provide a new target for clinical tumour therapy.

Current studies have indicated that autophagy acts as a 'double-edged sword' in cancer stem cells. In antitumour therapy, autophagic death can be induced in cells, and a variety of chemotherapeutic drugs achieve therapeutic effects through this route. In drug-resistant cancer stem cells, autophagy also appears to be a protective mechanism to reduce the killing effect of drugs on cells. The combination of autophagy inhibitors with traditional cancer treatment can induce the death of drug-resistant cancer stem cells to achieve better chemotherapeutic effects. An in-depth study on the regulatory mechanisms of autophagy in cancer stem cells may reveal a more valuable reference and therapeutic target for tumour therapy.

21.7 Antitumour Strategy for Targeting Autophagy in Cancer Stem Cells

Although there have been many reports on the research and clinical application of antitumour therapy and autophagy, some core problems remain controversial. Autophagy has a role in the promotion and inhibition of tumour development and metastasis processes. On the one hand, the tumour has not yet formed in the presence of damage, and autophagy is thought to prevent genomic instability or cell death and inflammation by inhibiting protein aggregation and eliminating damaged organelles and chromosome damage. It is theoretically feasible to prevent the occurrence of these injuries and ultimately inhibit the occurrence of tumours. Of course, it has been confirmed that autophagy is necessary for drugs to exert their therapeutic effects in the treatment of cancer. For example, mammalian target of rapamycin (mTOR) kinase inhibitors are used to treat renal cell carcinoma and metastatic breast cancer, and

inhibition of the mTOR kinase function produces an inhibitory effect on cell proliferation. As a central regulator of autophagy, mTOR kinase can inhibit the activation of autophagy. In addition, when the EGFR inhibitor drug erlotinib is used to treat lung cancer, autophagy is required to maximize its inhibitory effect. On the other hand, autophagy is a protective mechanism for cells in the face of harsh environments and has a certain role in promoting tumour development and metastasis. The rapid proliferative capacity of tumour cells requires more energy expenditure, but the efficiency of glycolytic metabolism is not high. Therefore, it is necessary to activate autophagy to produce more energy. Under this type of metabolic stress, autophagy inhibitors can induce tumour cell death. Imatinib has been studied as a means to block the fusion of autophagosomes and lysosomes in malignant glioma cells, increase mitochondrial damage, induce apoptosis and inhibit malignant glioma. The combination of chemotherapy drugs and autophagy inhibitors can enhance the effectiveness of tumour treatment. It has been found that the addition of the autophagy inhibitor 3-methyladenine (3-MA) or chloroquine (CQ) can enhance the apoptotic activity of human cervical cancer cells treated with cisplatin to improve the chemotherapeutic effect of cisplatin, and the use of chloroquine in bladder cancer cells can inhibit autophagy and activate apoptosis to enhance the sensitivity of tumour cells to radiotherapy (Wang et al. 2018). It has also been found that chloroquine potentiates the radiosensitivity of glioma-initiating cells by inhibiting autophagy and activating apoptosis (Ye et al. 2016). However, due to the lack of specific cell targets during autophagy therapy, autophagy also affects the function of normal cells and produces certain side effects. According to the theory of the origin of cancer stem cells, it is believed that cancer stem cells are critical for the malignant transformation, invasion and metastasis of tumours. Therefore, the combination of autophagy inhibitors and specific drugs that target cancer stem cells is expected to become more effective and produce fewer side effects than antitumour treatments.

21.8 Conclusions

Tumour stem cells are the key factors for tumorigenesis, growth, metastasis and chemoradiotherapy resistance. Cancer stem cells maintain the vitality of tumour cell populations through self-renewal and immortalization, and the migratory and invasive ability of cancer stem cells promotes the metastatic spread of the tumour. Cancer stem cells can be dormant for a long time and are not sensitive to external physical and chemical factors because of their drug resistance ability. Therefore, cancer stem cells are considered to be the keys to a breakthrough in cancer therapy. How to remove cancer stem cells or enhance their sensitivity to various treatments without affecting normal stem cells is a difficulty for tumour treatment in the future. It is especially important to understand the key mechanisms of tumour stem cell survival, migration and chemoradiotherapy resistance. Existing studies have shown that autophagy plays an important role in tumour stem cell survival and invasion and metastasis, and autophagy has different effects on tumour stem cells at different stages

of tumorigenesis and development. In the early stage of carcinogenesis and prior to the induction of malignant tumours, autophagy can inhibit the formation of tumour stem cells by eliminating damaged organelles and proteins and maintaining genomic stability. In the late stage of tumour development, autophagy can protect cancer stem cells in the harsh environment, such as that produced by ischaemia and hypoxia, to promote their survival and inhibit tumour stem cell apoptosis. However, does autophagy maintain the same status in different types of tumours? Is the heterogeneity of cancer still relevant? Autophagy can remove excess organelles, misfolded proteins and other waste materials in the cytoplasm. Autophagy can also provide the energy and substances cells need by degrading their own macromolecules. What is the regulatory role of autophagy in cancer stem cells? What are the similarities and differences between these various roles? These answers are still needed and require further study.

Although some existing results have shown a close relationship between autophagy and cancer stem cells, the importance of autophagy in cancer stem cells has started to emerge as a component of tumour prevention and treatment. However, most of the current related studies have been based on the observation of gene knockout animal models. Some autophagy-related genes are likely to have functions other than those related to autophagy. Therefore, when autophagy-related gene mutation analysis is used to study autophagy, phenotypic changes and other non-autophagy factors should be considered. The analysis of experimental results should also be considered to reach a conclusion. In addition, the current research on the relationship between autophagy and tumour stem cell characteristics mostly focuses on the observable phenomena, while the specific mechanism of autophagy regulation remains unclear. Further exploration of the role of autophagy during different life stages of stem cells is still urgent, and study of the molecular mechanism of autophagy regulation using modern biomedical research techniques is still necessary. These are important directions for research if autophagy is to serve as a powerful target for therapeutic applications in the future.

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Chapter 22
Autophagy and Tumour Metastasis

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Abstract Metastasis is the most important biological potential of malignant tumour cells. A variety of mechanisms is involved in regulating tumour invasion and metastasis and interacts with each other, forming a large regulatory system. Autophagy plays an important role in organisms in maintaining environmental homoeostasis. A large amount of evidence has shown that autophagy is also involved in tumour development processes, including invasion and metastasis. Autophagy not only controls some biological processes in tumour cells but is also affected by the microenvironment. Therefore, the role of autophagy in tumours is far more important and complicated than previously estimated. The role of autophagy in tumour metastasis will be discussed in this chapter.

Keywords Autophagy · Tumour · Metastasis · Microenvironment

Malignant tumours are one of the most serious abnormalities that threaten human health. Local infiltration and distant metastasis are the most important biological potentials of malignant tumour cells. Nearly 90% of cancer patients die from the distant metastasis of tumour cells to the main organs. The metastasis of tumour cells, which is a dynamic, continuous process that exists between tumour cells and host cells, is complex and multistep. A variety of mechanisms is involved in regulating tumour invasion and metastasis and interacts with each other, forming a large regulatory system. Scientists have continuously found many new important biological

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phenomena, such as the cancer stem cell theory and epithelial–mesenchymal transition, in the effort to gradually explore and reveal the potential mechanism of tumour cell metastasis.

Autophagy is a unique life phenomenon of eukaryotic cells that is widely distributed among eukaryotic cells. Autophagy is a process in which cells use lysosomes to degrade their damaged organelles and macromolecular substances, which is an important way for living organisms to maintain the balance of protein metabolism and stability of the intracellular environment. In recent years, a large amount of evidence has shown that autophagy participates in the regulation and control of all aspects of the invasion and metastasis processes, and this area has become a research field of concern to scholars worldwide. More importantly, tumour cells exist in a special microenvironment instead of in isolation. The metastasis of tumour cells is closely correlated with the tumour microenvironment and is regulated by various factors in the microenvironment. Autophagy not only controls some biological phenomena of tumour cells but also affects some functions of the tumour microenvironment. Therefore, the role of autophagy in tumours is far more important and complicated than previously estimated. The role of autophagy in tumour metastasis will be discussed in this chapter.

22.1 The Role and Mechanism of Autophagy in Tumour Invasion and Metastasis

Tumour metastasis refers to the process in which malignant tumour cells detach from the primary tumour and continue to grow after reaching discontinuous tissues or organs through various routes to form a secondary tumour with the same characteristics as the tissues in the primary tumour. The new tumours are called secondary tumours or metastases; this process is also called the invasion-metastasis cascade and involves several steps: (1) Local infiltration into the extracellular matrix (ECM) and interstitial cell layers; (2) Infiltration into the vasculature; (3) Survival in the vascular circulation; (4) Attachment to distant organs; (5) Extravasation into the parenchyma of distant tissues; (6) Survival in a microenvironment different from that of the primary focal micrometastases; (7) Restart of the proliferation mode to form larger metastases, which in turn develop into clinically detectable tumour foci (Fig. 22.1).

22.1.1 Local Invasion

Tumour cells leave their original tumour tissues and invade adjacent tissues, where they continue to proliferate. This process is called invasion. Tumour invasion includes a series of processes, for example, tumour cell adhesion, enzymatic degradation, migration and proliferation in the matrix. First, tumour cells need to destroy the

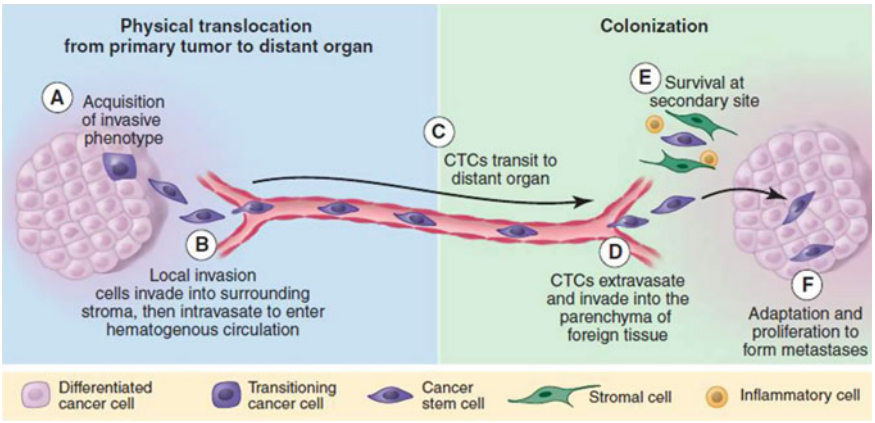

Fig. 22.1 The invasion-metastasis cascade of tumor

basement membrane and then enter the stromal tissue. There are several kinds of interstitial cells in the tumour stromal tissue, including fibroblasts, endothelial cells, macrophages and other immune cells, fat cells and mesenchymal stem cells. The interaction between these cells and tumour cells leads to increased aggressive tendencies in tumour cells.

22.1.2 Infiltration into the Vasculature

Endosmosis refers to the local invasion of cells into the lymphatic or vascular lumen. Although lymphatic metastasis of tumour cells can be observed in human tumours, tumour cells are mainly spread through the blood circulation. The occurrence of some molecular events can promote the penetration of tumour cells through the blood vessel wall composed of pericytes and endothelial cells into the bloodstream. In addition, endosmosis of tumour cells is also greatly affected by the vascular structure of tumour tissues. Tumour cells promote the formation of new blood vessels in their local microenvironment through VEGF production. Compared with the blood vessels in normal tissues, these new blood vessels have their own unique structural features, which are characterized by their meandering and tortuous appearance and by the weak connection between adjacent endothelial cells. The porous endothelial cells and permeable basement membrane create an advantageous condition for tumour cells to infiltrate.

22.1.3 Survival in the Circulatory System

When tumour cells successfully infiltrate the vascular lumen, they can spread through the venous and arterial circulation. These tumour cells are called circulating tumour cells (CTCs). CTCs need to surmount some stress conditions in the circulatory system and survive to reach distant metastases. The most prominent obstacle for CTC survival is the need to resist anoikis. Anoikis is one of the forms of programmed cell death; it is apoptosis induced by the detachment of normal epithelial cells from the ECM and plays an important role in maintaining the balance of growth, development and differentiation of normal tissue cells. In the blood circulation, although tumour cells are detached from the ECM, they have the ability to resist anoikis, survive and migrate to distant organs. In addition, CTCs need to overcome the damage caused by blood flow shear stress and the cytotoxic effect of immune cells, especially natural killer cells.

22.1.4 Extravasation of Blood Vessels

Upon reaching the microvessels of distant organs, CTCs can grow in the lumen of the blood vessels and form microclones, eventually eroding the vessel wall and allowing tumour cells to be in direct contact with the tissue parenchyma. Tumour cells may also adopt another approach—that is, penetrating the endothelial cells and the pericardial cell layer, migrating from the vascular lumen into the interstitium and connecting with the parenchyma. This process, which can be considered a reverse process of endosmosis, is called extravasation. Compared with blood vessels in tumour tissue, which are highly leaky during the infiltration process, blood vessels in normal tissue in the distal direction are normal during extravasation and thus have extremely low intrinsic permeability. To overcome this limitation, tumour cells destroy the microenvironment of the target organ by secreting factors and increasing the permeability of blood vessels.

22.1.5 Survival in the Unfavourable Environment of Target Organs, Forming Micrometastases

The environment encountered by tumour cells in the target organ is very different from the environment of the primary tumour; this difference is manifested in the type of interstitial cells, the components of the ECM, the types of growth factors and cytokines and the microstructure of tissues. Therefore, in the early stage in which tumour cells reach the target organ, they do not adapt to the microenvironment. However, some scholars have suggested that tumour cells can adapt to the harsh environment by changes in their own gene expression, and tumour cells also continuously transform the environment to promote the formation of metastases.

22.1.6 Formation of Metastases

The observation that tumour cells enter the target organ and survive does not imply continuous proliferation to form metastases. Cells may enter a long-term dormant state in which the number of cells does not increase or decrease; alternatively, due to environmental incompatibility, apoptosis continues to occur, offsetting cell proliferation and rendering metastasis formation difficult. Ultimately, only a very small number of tumour cells form clinically detectable metastases by improving their adaptability to the environment and activating self-renewal, successfully completing the invasion-metastasis cascade. Some evidence shows that this step is an important rate-limiting link in the invasion-metastasis cascade and plays a key role in tumour cell metastasis A Perspective on Cancer Cell Metastasis (Valastyan and Weinberg 2011).

22.2 Autophagy Promotes Tumour Invasion and Metastasis

Autophagy is a highly conserved process that is characterized by self-digestion and catabolism. It is one of the important mechanisms for maintaining cell homeostasis. As autophagy is a common biological phenomenon, the role of autophagy in the development of tumours has become a research area for scholars. In the early stages of tumorigenesis, autophagy can inhibit malignant transformation and tumour formation. In the late stage of tumour development, a large amount of evidence suggests that autophagy can promote tumour invasion and metastasis.

Autophagy can promote RAS-driven invasion. Knockout of autophagy-related genes can inhibit the invasion of epithelial cells overexpressing RAS in a three-dimensional culture system and can inhibit their migration in vivo and the formation of lung metastasis. Conditioned supernatant from normal cells with normal autophagy function can enhance the invasive ability of autophagy-deficient epithelial cells, indicating that autophagy-deficient cells are unable to secrete factors that promote invasion. Further studies have found that with the inhibition of autophagy, the secretion of the invasive factor interleukin-6 (IL-6) is decreased, and IL-6 supplementation in the cell culture system can restore the invasion level of RAS-overexpressing epithelial cells. In addition, the levels of matrix metalloproteinase 2 (MMP2) and WNT5A are reduced in autophagy-deficient cells. The results mentioned above indicate that the integrity of the autophagy pathway is essential for promoting the secretion of pro-invasion cytokines, including IL-6, and thus innovatively provide direct evidence for the critical role of autophagy in cell invasion. HMGB1 regulates the development of autophagy. Researchers found that miR-22 bound the 3' end of HMGB1, downregulated the expression of HMGB1, and thus inhibited HMGB1-mediated autophagy activation; moreover, miR-22 significantly inhibited the migration and invasion of osteosarcoma cells via autophagy inhibition.

Autophagy is involved in the extracellular delivery of many cytosolic proteins that do not pass through the Golgi apparatus into the traditional secretory pathway but are secreted directly into the cytosol in an unconventional manner. In mammalian cells, a typical example is the primary involvement of autophagy in the unconventional secretion of inflammatory cytokines such as IL-1β. However, studies have also found that the role of autophagy in the secretion of inflammatory cytokines may be more complicated. Autophagy plays an inhibitory role in the DNA damage-induced senescence-associated secretory phenotype (SASP) mainly by inhibiting the GATA-4 pathway, which is upstream of p62-dependent NF-κB. Therefore, determining how autophagy plays a role in these different cells to regulate the secretion of secreted factors is an approach for inhibiting invasion and metastasis in the future (Kang et al. 2015).

The quiescin sulfhydryl oxidase 1 (QSOX1) protein catalyses the formation of disulfide bonds and regulates protein folding and stability. Recent studies have shown that QSOX1 is associated with tumours. Researchers found that QSOX1 inhibits the proliferation, migration and invasion of breast cancer cells. Since oxidative stress and ER stress can induce the expression of QSOX1 and protect cells from death, oxidative stress and ER stress are closely related to autophagy, suggesting that QSOX1 may be involved in the regulation of autophagy. Under starvation conditions, QSOX1 expression is upregulated, preventing cells from entering autophagic death programmes and thus promoting cell survival. QSOX1 suppresses autophagy by inhibiting the fusion of autophagosomes and lysosomes. Further studies showed that inhibition of breast cancer cell invasion by QSOX1 was achieved by inhibiting autophagy, indicating that autophagy plays an important role in promoting tumour cell invasion. In a glioma cell line, shRNA was used to inhibit the autophagy-related gene Atg12, and it was found that cell activity, proliferation and migration were not affected; however, the 3D culture experiment showed that the cell invasion ability was significantly decreased. Lipopolysaccharide (LPS) can promote tumour invasion and metastasis. A study found that LPS activates TLR4 or TLR3 on lung cancer cells to induce autophagy, which in turn activates the MAPK and NF-κ B pathways by promoting the ubiquitination of TNF receptor-associated factor 6 (TRAF6). Both of these pathways can promote the production of IL-6, CCL2/MCP-1, CCL20/MIP-3α, VEGFA and MMP2. These factors are necessary to activate TLRs to promote the migration and invasion of lung cancer cells. These results demonstrate that autophagy activated by TLR4 and TLR3 is an important mechanism mediating invasion and metastasis in lung cancer, suggesting that the inhibition of autophagy can be a potential target for lung cancer treatment.

In recent years, the “cancer stem cell (CSC)” hypothesis, which defines tumour stem cells as “cells with self-renewal ability in tumour tissues that simultaneously produce tumour cells with different degrees of differentiation”, has been proposed in cancer research. CSCs have been detected and isolated in various tumours, such as leukaemia, skin cancer, breast cancer, glioma, pancreatic cancer, colon cancer and liver cancer. This hypothesis holds that cancer stem cells are closely related to the occurrence, invasion and metastasis of tumours. As mentioned above, tumour

cells need to undergo a complex, multilevel invasion-metastasis cascade to complete the metastasis process and form metastases. However, not all tumour cells have the ability to successfully complete this cascade. Only a very small number of CSCs with strong self-renewal ability can eventually reach the target organ and form metastases. Studies have shown that autophagy is involved in the regulation of CSC stemness maintenance and survival. Silencing autophagy-related gene expression, using autophagy inhibitors or siRNA, results in a complete clearance of almost all leukaemia CSCs. Our previous study found that the autophagy level of CD133+ CSCs in liver cancer cells is higher than that in CD133- hepatoma cells. The use of autophagy inhibitors can inhibit the suspension and formation of CD133+ CSCs in tumours and tumourigenicity in vivo, and autophagy can promote the survival of CD133+ CSCs under hypoxic and nutrient-insufficient conditions. The above evidence suggests that autophagy can promote tumour invasion and metastasis by regulating CSCs.

During the process of autophagy, the proteins and other components that need to be degraded are encapsulated in a single- or double-layer plasma membrane to form autophagosomes, which then fuse with lysosomes to form autophagosomes, which degrade the contents. The degradation of protein polymers by autophagy requires the involvement of ubiquitin and the ubiquitin-binding receptor P62 protein. P62 also binds to ubiquitinated proteins and the autophagosome membrane protein LC3, thereby transferring ubiquitinated proteins to autophagosomes and initiating autophagic degradation. This degradation pathway is an important pathway for the degradation of long-lived intracellular proteins and organelles, but there is increasing evidence that it has become a pathway for most intracellular protein degradation. Therefore, autophagy may regulate invasion and metastasis by degrading related protein molecules that regulate tumour cell invasion and metastasis. For example, it has been found that the expression of PEDF, a tumour suppressor gene, is decreased in highly invasive melanoma tissues. Exposure of melanoma cells to hypoxia in vitro can inhibit the expression of PEDF and promote their invasiveness, and it was found that this inhibitory effect is mediated by hypoxia-activated autophagy and enhances the degradation of PEDF.

22.3 Mechanism of Autophagy in Promoting Tumour Invasion and Metastasis

22.3.1 Autophagy Enhances the Tumour Stem Cell Phenotype

Tumour cells undergo epithelial–mesenchymal transition (EMT), in which adherent epithelial cells transform into highly mobile stromal cells. This process is an important step in the early stages of tumour invasion and metastasis and involves

cytoskeletal remodelling; downregulation of connexin, which maintains cell connections; and upregulation of interstitial proteins in epithelial cells. These events result in the loss of cell polarity, opening of cell–cell junctions, degradation of the ECM, and secretion of various matrix metalloproteinases.

Studies have shown that both EMT and autophagy promote the role of cancer stem cells, which links EMT to autophagy (May et al. 2011; Aguilar et al. 2016). EE Mowers et al. reported that autophagy promotes metastasis by inducing EMT, relying to some extent on the regulation of EMT by TGF-β. Studies have also shown that ULK2 promotes autophagy activation by phosphorylating a starting complex containing Beclin1, downregulating E-cadherin and increasing invasiveness. In glioblastoma, autophagy enhances the stemness phenotype of predominantly mesenchymal cells, thereby conferring invasion and metastasis characteristics on glioma stem cell lines. Cancer stem cells are thought to drive tumour cell metastasis because of their motility, plasticity, and ability to multiply new tumour stem cells and produce heterogeneity at secondary tumour sites. Many clinical reports have also confirmed the relationship between stemness-related markers and invasion (Marquardt et al. 2018; Munro et al. 2018). Induction of EMT promotes the tumour stem cell phenotype by activating transcription factors. For example, the zinc finger transcription factor Slug is activated, which regulates the involvement of E-cadherin in EMT, activates the expressions of self-renewal genes, and upregulates CD44 expression and promotes tumour proliferation in breast cancer cells.

Autophagy is equally important for maintaining the stemness of $CD44^+/CD24^-$ breast cancer stem cells. The main promoter of autophagy, Beclin1, was screened from an shRNA gene library as a regulator of the plasticity of cancer stem cells. Under normal circumstances, autophagy plays important roles in maintaining the function of normal tissue stem cells, such as maintaining the survival of haematopoietic stem cells and inhibiting ageing of muscle stem cells. In human breast ductal carcinoma in situ (DCIS), a small population of cells with an initial ability for invasion and metastasis exhibits enhanced autophagy, and their survival and maintenance of the stem cell phenotype are dependent on autophagy. The role of autophagy in $CD44^+/CD24^-$ breast cancer cells was further confirmed. ATG4 (autophagy-associated gene 4) regulates cell populations, forms mammary glands in vitro and forms tumours in vivo. Therefore, in the new model, cancer stem cells combined with autophagy can induce EMT, promote self-renewal and increase migration capacity, survival and drug resistance under hypoxic or stress conditions (Cufi et al. 2011).

22.3.2 Autophagy Regulates Cell Adhesion Kinetics

Cell migration is an important condition for tumour growth and invasion. The main process in migration is cell polarization, during which the membrane protrudes from the pseudopod, cellular actin begins to contract, and cells move on the adherent plaque and are ultimately separated from the base. Focal adhesions are macromolecular protein complexes associated with the plasma membrane of cells, which are linked to

the inside and outside of cells by integrins. Integrins act as a bridge between cells and the extracellular matrix and play an important role in cell proliferation, tumorigenesis and metastasis. The distribution of integrins in cells is not stable; integrins undergo internalization, recycling and degradation, and transport, while the turnover of focal adhesions and cell migration are regulated by integrin transport.

Integrins participate in the synthesis of focal adhesions and promote signalling and functional expression supporting cell migration. It has been demonstrated that under the induction of cell tension, the extracellular matrix acts as a ligand to bind to integrins, which causes changes in the cell tail region, promoting the recruitment of the adaptor proteins TLN (prion protein) and PXN (plycan). Upon tension increase and focal adhesion maturation, integrins recruit PTK2 (protein tyrosine kinase 2) and the non-receptor tyrosine kinase SRC, promoting downstream signalling pathways such as Rho GTPase signalling, anoikis signalling and mitotic signalling; moreover, cellular pathways such as matrix transport signalling are activated (Ganguly et al. 2013; Seguin et al. 2015).

Recent studies have confirmed that autophagic degradation of adhesion proteins promotes cell migration. Autophagy degrades adhesion proteins to facilitate disassembly and turnover, whereas loss of autophagy reduces cell migration and metastasis. After knockout of the autophagy genes ATG7, ATG12 and ATG5, the volume and number of adhesion proteins were increased, as evidenced by PXN and AYX assays. Adhesion proteins and assembly rates were assessed by fluorescently labelled PXN assays, and it was found that the rates of assembly and disassembly of adhesion proteins were significantly reduced in autophagy-deficient cells. Autophagy targets binding to adhesion protein complexes such as NBR1, PXN and SRC p-Y416 to facilitate their degradation. NBR1 is a selective autophagy cargo receptor; NBR1 recruits autophagosomes to adhesion proteins in MCF10A cells that undergo HRAS transformation. Inhibition of NBR1 reduces the turnover of adhesion proteins and cell migration. However, inhibition of NBR1 in other breast cancer cell lines had no effect on migration, suggesting that NBR1 is not necessary for the disassembly of adhesion proteins in all cell types.

Overexpression of the SRC protein kinase is involved in cell metastasis in solid tumours. In squamous cell carcinoma, Sandilands E et al. found that the autophagy-associated proteins ATG7, ATG12 and LC3 co-localize in cell vesicles with SRC and SRC p-Y416. Since the ATG protein is involved in the formation of autophagosomes, SRC is hypothesized to be a target protein for autophagic degradation in the case of concomitant autophagy and SRC expression. Subsequently, Sandilands E et al. confirmed the relationship between autophagy and adhesion proteins in co-immunoprecipitation experiments with SRC p-Y416, total SRC and LC3 and found that disrupting adhesion activity after PTK2 knockout can increase CBLC-mediated colocalization of LC3, ATG7 and SRC in the cytoplasm. In addition, Plaza-Menacho I et al. found that autophagic targeting of SRC can promote the degradation of the RET receptor tyrosine kinase, an adhesion plaque-associated kinase. Autophagy and adhesion plaque signalling pathways are linked by targeting the effects of SRC p-416 and RET, suggesting that autophagy-mediated degradation of SRC contributes to the

disassembly of adhesion plaques and thus promotes cell migration (Kenific et al. 2016; Sharifi et al. 2016; Mowers et al. 2016).

FAK (an adhesion plaque kinase) and SRC (a protein tyrosine kinase) are important regulatory molecules in adhesion kinetics. FIP200 is a focal adhesion kinase family interacting protein that binds to FAK and inhibits its activity. It has also been found that FIP200 is an important player in the formation of ULK complexes during the initiation of autophagy. High expression of FIP200 inhibits FAK autophosphorylation, thereby inhibiting the cell cycle and attenuating migration. In a nutrient-deficient environment, by direct activation of the AMPK signalling pathway or indirect action on mTOR through TSC2 and inhibition of its activity, ULK activity is increased, and ULK complex phosphorylation promotes autophagy. In an in vivo model, inhibition of AMPK and ULK activity by HSP90 (Hsp90) resulted in FK200-mediated inhibition of FAK, thereby promoting cell invasion and metastasis (Chen et al. 2016; Mowers et al. 2017).

22.3.3 Autophagy Induces Epithelial–Mesenchymal Transition in Tumour Cells

There are tight junctions between epithelial cells, which attach epithelial cells in the tissue and limit their movement. Interstitial cells have a long, spindle-like shape that maintains a limited connection with the surrounding cells; therefore, these cells have high-mobility. Epithelial–mesenchymal transition (EMT) is a phenomenon in which epithelial cells acquire a mesenchymal phenotype and transform into mesenchymal cells. The concept of EMT was first proposed in 1982. Researchers found that lens epithelial cells can temporarily lose cell polarity, transform into a mesenchymal cell-like morphology in collagen gels, and acquire migration ability, and thus proposed EMT. EMT is essential at an important stage in the embryonic development of many species and plays an important role in the formation of various organs and tissues, including the formation of the neural crest. Recent studies have found that EMT plays an important role in the early stages of tumour metastasis. The expression of cell adhesion molecules such as E-cadherin in tumour cells is downregulated or abrogated, the actin-based cytoskeleton is transformed into a vimentin-based cytoskeleton, vimentin expression is upregulated and cells become interstitial. Cell morphology is altered and cells gain a strong capacity for motility, which promotes local invasion and invasion of the vasculature. Transcription factors such as Slug, Snail, Twist and ZEB1 can inhibit the expression of E-cadherin and induce the expression of interstitial markers, which play a key role in the regulation of EMT. TGF-β is currently recognized as one of the cytokines that induces EMT in tumour cells. TGF-β can upregulate the expression of transcription factors such as Snail, ZEB1 and ZEB2 through the receptor-activated Smad signalling pathway and promote the occurrence of EMT in tumour cells.

After knockout of autophagy-related genes, real-time quantitative PCR was used to detect the expression changes of EMT-related genes in breast cancer cell lines, and it was found that the expression of vimentin was decreased at the transcriptional level. Inhibition of ATG12 expression by shRNA or use of the autophagy inhibitor chloroquine can inhibit the TGF-β1-induced expression of vimentin, suggesting the positive regulatory effect of autophagy on EMT. Hank's balanced salt solution was used to generate a starvation condition for the culture of the hepatoma cell lines HepG2 and BEL7402. It was found that EMT occurred in hepatocarcinoma cells, the expression of the epithelial markers E-cadherin and CK18 was downregulated, the expression of the mesenchymal marker fibronectin was upregulated, and the expression of the invasion-related protein MMP-9 was upregulated. However, if siRNA was used to inhibit Atg3 or 7, this change was not observed, indicating that the EMT process induced in hepatocellular carcinoma cells by starvation conditions is mediated by autophagy. Further studies found that autophagy-mediated EMT is achieved by promotion of TGF-β expression and Smad3 pathway activation. This study revealed a possible mechanism by which autophagy regulates tumour EMT, invasion and metastasis. Intermittent hypoxia can promote the expression of HIF-1α and activate autophagy, enhance the invasive ability of pancreatic cancer cells, and induce tumour cells to acquire the phenotype of cancer stem cells. By the detection of molecular markers of EMT, it was found that intermittent hypoxia can induce EMT in pancreatic cancer cells. The use of siRNA and autophagy inhibitors can reverse the increase in EMT and the invasion ability induced by intermittent hypoxia. This finding also provides evidence for the role of autophagy in hypoxia-promoted tumour EMT, invasion and metastasis.

It is well known that downregulation of the epithelial cell marker E-cadherin (CDH1) is a key molecular event in the development of EMT. In the invasive area at the junction of advanced tumour tissue and normal tissue, the expression of E-cadherin in tumour cells is absent or decreased; knocking down E-cadherin expression can promote EMT in tumour cells and obtain high invasiveness. There is evidence that E-cadherin can be degraded by lysosomes after ubiquitination. Therefore, whether autophagy may promote EMT and enhance invasion and metastasis of tumour cells by degrading E-cadherin is worthy of further study.

22.3.4 Autophagy Can Inhibit Apoptosis Caused by Anoikis During Metastasis

Anoikis is one of the forms of programmed cell death. Anoikis is apoptosis induced by the detachment of normal epithelial cells from the extracellular matrix (ECM) and plays an important role in maintaining the balance of growth, development and differentiation of normal tissue cells. When cells acquire the characteristics of migration and proliferation in an unsuitable new environment they lose normal cell–matrix junctions when the cell cycle is arrested, and caspase-mediated programmed death,

apoptosis, is initiated. In this situation, apoptosis is anoikis. In aphakia, apoptosis can clear ectopic cells and prevent abnormal growth.

Compared with normal epithelial cells, tumour cells are not sensitive to anoikis and can survive and proliferate without adhering to the ECM, which is important for the successful migration of tumour cells. The occurrence of anoikis is not limited to only when cells are detached from the ECM, also occurs due to ECM binding in ectopic tissues. Therefore, in each step of the metastasis cascade, from local invasion and migration through the circulatory system to implantation in the target organ, tumour cells need to resist anoikis and proliferate.

Research evidence suggests that autophagy is activated when cell nests are lost and is involved in regulating tumour cell resistance to anoikis. In suspension culture, the number of GFP-LC3 puncta increased in MCF10A human mammary epithelial cells and immortalized mouse embryonic fibroblasts that overexpressed the Ras gene, LC3II expression increased and p62 degradation increased, suggesting that autophagy was activated. In three tumour cell lines (MDA-MB-231 breast cancer cells, HCT-116 colon cancer cells and PANC-1 pancreatic cancer cells) with K-Ras activating mutations, it was also found that non-adherent culture activated autophagy. Further studies revealed that the expression of mTORC1, a negative regulatory molecule of autophagy, is downregulated in suspension culture, thereby mediating the activation of autophagy. EGFR is critical for the survival and proliferation of epithelial cells, and its expression is significantly decreased when nests are lost. Autophagy occurs when EGF is downregulated, while overexpression of EGFR can effectively inhibit autophagy caused by nest loss.

In the case of nest loss, the signal to activate autophagy includes the following aspects. The first signal operates through growth factors and nutrient-sensing pathways, and the normal function of cell surface growth factor receptors requires integrin-mediated cell adhesion. For example, EGFR expression is decreased when many epithelial cell integrin adhesions are lost. Expression of cell surface receptors or growth factor receptors via nutritional regulation results in potent growth-promoting pathway inactivation, primarily, the mammalian target of rapamycin (mTOR) pathway. Correspondingly, mTOR expression is also decreased during nest loss; since mTOR is a negative regulatory molecule of autophagy, autophagy is activated when nest loss occurs. Focal adhesion kinase (FAK) is a key molecule in signalling pathways mediated by adhesion and can phosphorylate the mTOR upstream regulatory molecule tuberous sclerosis complex 2 (TSC2), thus inhibiting its activity and thereby maintaining mTOR in an active state. Inhibition of FAK inhibits mTOR activity, and this pathway provides a direct link for the association between integrins and autophagy. The energy-related signalling pathway is also involved in the activation of autophagy. AMP-activated protein kinase (AMPK) can detect a decrease in energy (a decrease in ATP levels). In the absence of energy, due to the increased AMP/ATP ratio, AMPK is phosphorylated by its upstream kinase LKB1 and activates the TSC1/2 complex, which in turn inhibits the downstream molecule mTOR, not only inhibiting growth-promoting signalling but also inducing autophagy to provide ATP. In addition, autophagy can be activated not only by metabolic stress but also by oxidative stress; an increase in ROS levels may be an important signal for

activation of autophagy in catastrophic cells, which provides another clear line of evidence that nest loss can activate autophagy.

Cells activate autophagy to activate lysosomal processes in order to counter metabolic stress. Nest loss causes cell starvation. Autophagy is a temporary method to provide energy for tumour cells, delay the onset of apoptosis and provide the opportunity for cells to survive. Once tumour cells have the opportunity to reattach to the ECM, they can survive. In addition, aerobic glycolysis is a decisive feature that supports malignant transformation and the high proliferation rate of tumour cells. In cells overexpressing Ras, anoikis-induced autophagy promotes the occurrence of aerobic glycolysis and promotes cell survival and proliferation. A series of experiments by Debnath showed that autophagy promotes cell survival under nest loss conditions. These researchers used a special 3D cell culture model to simulate anoikis. The results in various cell lines showed that nest loss quickly induced the occurrence of autophagy, inhibited autophagy-induced cell survival and increased apoptosis. These results provide a possible explanation for the resistance of tumour cells to anoikis and provide a promising therapeutic target. However, the observation that autophagy promotes tumour cell metastasis by inhibiting anoikis also needs to be verified in an in vivo model.

Recent studies demonstrated that autophagy inhibits apoptosis caused by anoikis by a variety of mechanisms. One study found that autophagy promotes the phagocytosis of damaged mitochondria to maintain cell homeostasis, which is the initiation of cell-protective mechanisms in response to oxidative stress, promotes cell adaptation to stress, and ultimately promotes cell survival in the absence of nests (Hawk and Schafer 2018; Satyavarapu et al. 2018). Avivar-Valderas A. et al. demonstrated that the ROS-dependent protein kinase R-like endoplasmic reticulum kinase (PERK) is activated by autophagy in response to anoikis or integrin blockade in a breast tumour model. In addition, inhibition of PERK or of autophagy itself can promote cell death and reduce clonality. This experiment confirmed the role of PERK-induced autophagy in tumour cell anoikis. In addition, these researchers found that detachment of mammary epithelial cells from the matrix induces autophagy gene expression by activating the EIF2AK3-ROS-ATF4 signalling axis and enhances anoikis resistance. EIF2AK3 also activates AMPK and inhibits MTORC1-SERPINE1 signalling, promoting autophagy-mediated anoikis. This study demonstrated that autophagy, as a pro-survival mechanism, may promote EIF2AK3-ATF4-mediated upregulation of autophagy gene expression and that EIF2AK3-mediated inhibition of MTORC1 promotes matrix separation (Avivar-Valderas et al. 2011). Another study reported that inhibition of autophagy can promote anoikis in hepatocarcinoma cells in suspension culture. In mice intraperitoneally injected with hepatocarcinoma cells, hepatoma cells with ATG gene silencing could not form abdominal metastases, suggesting that the inhibition of autophagy can be relieved. The inhibition of autophagy can reduce anoikis resistance and inhibit the metastasis of liver cancer cells. Further mechanistic studies found that after autophagy inhibition, the protein levels of the proapoptotic genes BAX, BAK1 and FADD increased, while protein expression of the anti-apoptotic gene BCL2L1 decreased, indicating that the inhibition of autophagy may also promote anoikis by regulating the apoptosis signalling pathway.

22.3.5 Autophagy Provides Energy for Tumour Cell Invasion and Metastasis

Chronic uncontrolled cell proliferation is one of the most important features of tumours. The energy metabolism of tumour cells changes to support sustained cell growth and division, thereby promoting tumour invasion and metastasis. Under aerobic conditions, normal cells convert glucose to pyruvate by aerobic oxidation, followed by pyruvate oxidation in mitochondria to form carbon dioxide through the tricarboxylic acid cycle, a process known as oxidative phosphorylation. Under hypoxic conditions, cells initiate anaerobic glycolysis. Tumour cells convert glucose to lactic acid by glycolysis in the presence of oxygen through a process called aerobic glycolysis. This phenomenon was proposed by Otto Warburg in the early 1920s and is known as the Warburg effect.

Cancer-associated fibroblasts (CAFs) are important mesenchymal cells in the tumour microenvironment. Lisanti et al. found that the expression of Caveolin-1 (Cav-1) in breast cancer CAFs was unchanged at the transcriptional level but was significantly decreased at the protein level. Mammary mesenchymal fibroblasts obtained from Cav-1 knockout mice have the same characteristics as human CAFs, including high proliferation, increased collagen production, activation of TGF-β signalling, high expression of muscle-related genes, and contractility) and genome-wide expression analysis showed similar transcriptional expression characteristics.

Studies have found that in interstitial cells with Cav-1 deletion, the levels of a series of glycolytic marker molecules such as PKM2, LDH, enolase and acid aldolase A are elevated, suggesting that the Warburg effect may occur in the tumour stroma, not in epithelial tumour cells. Consistent with these findings, the mitochondrial dysfunction marker BNIP3L and the L-lactic acid production and secretion marker monocarboxylate transporter 4 (MCT4) were also positively expressed in Cav-1-deficient mesenchymal cells. It was observed under electron microscopy that a large number of lysosomes and autophagosomes in CAFs contained degraded mitochondria-like structures, while breast cancer cells contained a large number of normal mitochondria. Based on substantial evidence, Dr. Michael P. Lisanti and his colleagues proposed the reverse Warburg effect in 2009, in which tumour-associated fibroblasts convert glucose into L-lactic acid by glycolysis and become a factory for this high-energy metabolite. Lactic acid is transported to tumour cells, and a large amount of ATP is produced by the tricarboxylic acid cycle and aerobic mitochondrial metabolism. Tumour cells parasitize adjacent mesenchymal cells and use them as a source of energy.

The specific mechanism through which tumour cells utilize energy is that they generate ROS, causing oxidative stress in CAFs, which leads to mitochondrial dysfunction and a decrease in Cav-1 expression. This initial event further promotes the production of additional ROS by CAFs, and the increase in interstitial ROS can lead to DNA damage and polyploidy in tumour cells. Mitochondrial dysfunction in CAFs upregulates HIF-1α and NF-κB, which in turn causes autophagy and mitophagy, activates the reverse Warburg effect, and provides a nutrient-rich microenvironment

for tumour cells. In addition, tumour cells protect themselves from oxidative damage by upregulating the anti-apoptotic protein TIGAR and the antioxidant enzyme peroxiredoxin-1. Moreover, autophagy results in Cav-1 degradation in autophagic lysosomes, further affecting this process. The application of chloroquine, a lysosomal inhibitor, can inhibit the loss of Cav-1, suggesting that the downregulation of Cav-1 expression is due to lysosomal degradation during autophagy.

The expression of autophagy-related molecules and lysosomal enzymes in Cav-1-deficient mesenchymal stem cells is significantly increased, a condition that was also confirmed in human tumour stromal tissues. It is suggested that the tumour cells can bypass parasitism and that their anabolic needs can be satisfied by catabolism of the tumour stroma; this model is called the autophagic tumour stroma model. These findings suggest that the inhibition of autophagy in the tumour stroma inhibits tumour growth. Moreover, the induction of autophagy in tumour cells inhibits tumour growth, since the activation of autophagy prevents tumour cells from utilizing the nutrients provided by the stroma. The results mentioned above can explain the paradox that both the inhibition and induction of autophagy can inhibit tumour growth.

Further studies have confirmed that autophagy may regulate energy metabolism by affecting the tumour microenvironment. The metabolites lactic acid and ketone, secreted by tumour-associated fibroblasts, promote the anabolism and survival of tumour cells. It has recently been found that overexpression of autophagy genes such as BNIP3 or ATG16L1 in human fibroblasts enhances autophagic flux and increases the production of lactic acid and ketone bodies. Compared with the injection of fibroblasts transfected with empty vector, co-injection of fibroblasts and breast cancer cells overexpressing ATG16L1 or BNIP3 into nude mice resulted in increased colonization of lung metastases in nude mice, indicating that autophagic activity in CAFs can promote the development of tumours. In contrast, overexpression of ATG16L1 in breast cancer cells can inhibit tumour growth in nude mice, suggesting that autophagy plays an important role in tumour progression. Oxidative stress in the tumour microenvironment also drives autophagy-mediated metabolic coupling. Autophagy, glycolysis and expression of the lactate receptor SLC16A4 are induced in CAFs by the HIF1α and NF-κB signalling pathways to promote xenograft tumour growth. TGFβ1 and its downstream target gene CTGF also activate autophagy and stromal cell catabolism, driving metabolic coupling and xenograft tumour growth. Interestingly, autophagy induced by TGFβ1 and HIF1α in MDA-MB-231 cells inhibited the growth of xenograft tumours, further confirming that autophagy may play different roles in the environment of the stroma and tumour cells (Avivar-Valderas et al. 2011; Chiavarina et al. 2010).

22.3.6 Autophagy and Tumour Cell Dormancy

Disseminated tumour cells (DTCs) can survive in distant target organs for many years without forming metastases. Dormant tumour cells are often undetectable at

diagnosis and are resistant to conventional chemoradiotherapy for primary proliferating tumour cells. Therefore, exploring the mechanisms that regulate dormancy is important for developing treatments to combat DTCs.

DTCs are unable to form a strong connection with the ECM in a new environment when transferred to distant target organs; thus, dormancy is induced. Inhibition of the β1 integrin signalling pathway in breast cancer models is recognized to induce not only autophagy but also dormancy. Both in vitro and in vivo experiments have shown that interfering with β1 integrin specifically activates cell dormancy. Inhibition of β1 integrin activity inhibits tumour cell proliferation but does not affect cell viability, allowing cells to enter a dormant state. It is, therefore, speculated that when DTCs are in a new environment, autophagy is activated to promote their survival and entry into a dormant state. In addition, dormant cells need to resist apoptotic signals in the environment. During the process of breast cancer cell metastasis to bone, DTCs can exist in a dormant state in the bone marrow for an extended duration. TRAIL is abundant in the bone marrow microenvironment and kills dormant cells. However, a mechanism involving Src can promote the resistance of DTCs to TRAIL-induced apoptosis and ensure their survival. Since autophagy protects cells against TRAIL-induced apoptosis, it can be speculated that protective autophagy will initiate dormancy in DTCs and promote their survival in the bone marrow.

Recent reports in ovarian cancer provide direct evidence of autophagy and tumour cell dormancy. The tumour suppressor gene aplasia Ras homolog member I (ARHI) activates autophagy and promotes the survival of dormant cells in the tumour microenvironment in vivo. Further research is needed to explore the regulatory mechanism of autophagy in the existing dormancy model as well as to investigate the effect of gene interference with ATG expression, the effect on dormancy and whether autophagy initiates the death of dormant tumour cells, which will provide a new target for tumour therapy.

In addition, autophagy can promote the final step in the completion of the transfer cascade by promoting the growth of tumour cells in distant metastases and the ultimate achievement of a successful transfer. Jia Fan et al. used immunohistochemical staining to find that the expression of LC3 was higher in liver cancer metastases than in primary tumours, indicating that autophagic activity was high in liver metastases. Furthermore, in a mouse lung metastasis model, the LC3 level in the metastatic clones was higher than that in the primary tumour and was highest in the early metastatic clones; dynamic monitoring showed that there was no significant change in the level of autophagy during cell migration, invasion or anoxia. It is suggested that autophagy may promote the metastasis of liver cancer by promoting the implantation of liver cancer cells in distant organs.

22.4 The Role of Microenvironmental Factors that Induce Autophagy in Tumour Invasion and Metastasis

22.4.1 The Role of the Hypoxic Microenvironment in Tumour Invasion and Metastasis

The ischaemia and hypoxia caused by insufficient blood supply is a very prominent feature of the tumour microenvironment. During the period of rapid tumour growth, the formation rate of new blood vessels is insufficient to meet the needs of rapidly proliferating tumour tissues, resulting in insufficient local blood supply to the tumour and forming an ischaemic and hypoxic microenvironment. In the early stage of tumorigenesis, as well as in the processes of metastasis and recurrence of tumour metastasis, an ischaemic and hypoxic microenvironment is also present. This ubiquitous microenvironment with a low blood supply can cause corresponding biological changes in tumour cells to enhance adaptability to this microenvironment, and in this process, tumour cells acquire enhanced invasion and metastasis abilities. These enhanced abilities induce tumour cells to leave the primary tumour and implant in the target organ. Hockel et al. found that patients with severe tumour hypoxia had significantly shorter survival times than those with a better oxygen supply. A mouse model was used to find that hypoxia caused by surgery can promote the dedifferentiation of residual tumour cells into CSCs, thereby promoting the formation of metastases. Hypoxia can inhibit the expression of E-cadherin in tumour cells, induce EMT, and promote the invasion and metastasis of tumour cells.

Hypoxia-inducible factor-1α (HIF-1α) is a key transcription factor regulating the tumour hypoxia response. In normal oxygen concentrations, HIF-1α can be rapidly degraded by proteases, and its half-life is less than 5 min. In hypoxia, its half-life is prolonged, and it can bind to HIF-1β to form HIF-1 transcription factors and regulate gene expression at the transcriptional level. As a functional subunit of HIF, HIF-1α is the rate-limiting factor of HIF-1 activity and the only oxygen-modulating subunit. All HIF-l-regulated target genes contain a hypoxia response element (HRE). HIF-1 specifically recognizes and binds to the core HRE sequence, 5'-RCGTG-3', which induces the transcriptional activation of target genes, causing a series of cell responses to hypoxia. Many studies have confirmed that HIF-1 can upregulate the expression of VEGF and other factors, promote the growth of vascular endothelial cells, and enhance the permeability of blood vessel walls, thereby increasing the supply of nutrients and oxygen to tumour cells, and providing more convenient conditions for the metastasis of tumour cells. HIF-1α activates the expression of transcription factors such as Snail and twist and induces EMT in tumour cells; in addition, it promotes the gene transcription and expression of cathepsin D and MMPs, degrades the extracellular matrix, and promotes tumour invasion and metastasis.

Hypoxia is an important factor that can induce the occurrence of autophagy. Studies have shown that hypoxia-induced autophagy in cells is closely related to HIF-1. In the early stages of hypoxic stimulation, cells upregulate HIF-1 and initiate

the expression of a series of downstream genes to protect themselves from damage caused by hypoxia. HIF-1 is a core transcription factor that regulates the oxygen balance in living organisms and plays a central role in the signalling pathway of hypoxia. HIF-1 can regulate hypoxia-induced autophagy by regulating the expression of the downstream target molecules BNIP3 and BNIP3L. BNIP3 induces autophagy by interfering with interactions between beclin-1 and bcl-2 and bcl-xl. Experimental evidence shows that under hypoxic culture conditions, the invasive and metastatic abilities of various tumour cells are enhanced, and autophagy in tumour cells is activated. The use of siRNA or autophagy inhibitors can significantly inhibit the invasion and metastasis of tumour cells under hypoxic conditions, indicating that the hypoxia-induced invasion and metastasis of tumour cells is dependent on the activation of autophagy.

22.4.2 The Role of the Inflammatory Microenvironment in Tumour Invasion and Metastasis

Many studies have shown that there is a close relationship between chronic inflammation and tumorigenesis. Epidemiological surveys show that colon, prostate, liver, pancreatic and other tumours are often associated with chronic inflammation, and long-term use of non-steroidal anti-inflammatory drugs can significantly reduce the incidence of colorectal cancer, lung cancer, stomach cancer, oesophageal cancer and breast cancer. In addition to ischaemia and hypoxia, substantial infiltration of inflammatory factors is the most prominent feature of the tumour microenvironment. Leukocyte infiltration and cytokine and chemokine secretion are key factors in tumour-associated inflammation. It is well known that tumour-associated macrophages (TAMs) and their secreted factors, such as IL-1 and TNF-α, can promote almost all aspects of tumour invasion and metastasis. In vivo imaging experiments in mice showed that tumour cells migrate through EMT and that this process is dependent on the inflammatory microenvironment generated by TAMs and other mesenchymal cells, such as CAFs. Recently, it has been reported that TNF-α can increase the activity of the Snail promoter and promote EMT in breast cancer cells. This finding provides more powerful evidence for the association between inflammation and EMT. In addition to directly inducing EMT, TNF-α can upregulate the expression of TGF-β, which significantly promotes the induction of EMT by TGF-β. Snail, in turn, promotes the expression of the pro-inflammatory factors IL-1, IL-6 and IL-8, suggesting that the interaction between inflammation and EMT plays an important role in tumour progression. Other studies have shown that there is also a correlation between inflammation and autophagy. TNF-α-mediated upregulation of NF-κβ not only promotes tumorigenesis but also upregulates the expression of the autophagy gene Beclin1. In a study of human hepatoma cells and breast cancer cells, it was found that the inflammatory factor TGF-β can promote the expression of the autophagy genes Beclin 1, ATG5 and ATG7, thereby activating autophagy. The autophagy gene

ATG16L1 plays a role in the control of inflammatory immune responses and the maintenance of the intestinal barrier, both of which are important for preventing the development of intestinal inflammation. ATG16L1 deletion induced the inhibition of autophagy, which enhanced endotoxin-induced inflammatory immune responses. Collectively, these results indicate that inflammation may have a regulatory effect on autophagy. Our previous study found that mesenchymal stem cells pretreated with the inflammatory factors IFN-γ and TNF-α exhibit increased secretion of TGF-β, which promotes EMT in hepatoma cells and activates autophagy in hepatoma cells. This finding shows the promotive effect of mesenchymal stem cells in the inflammatory tumour microenvironment on invasion and metastasis in hepatocarcinoma and suggests that autophagy may play a certain role in these processes. In addition, the immune system regulates tumour metastasis through cytokine-induced apoptosis, especially apoptosis mediated through TNF-related apoptosis-inducing ligand (TRAIL), which plays a key role in T cell and NK cell inhibition of tumour metastasis. TRAIL receptor mutations render tumour cells resistant to TRAIL-induced apoptosis, resulting in increased metastasis. Interestingly, recent studies found that autophagy is activated in tumour cells that are resistant to TRAIL, which acts to protect tumour cells, as the inhibition of autophagy promotes apoptosis. Therefore, in addition to gene mutation in TRAIL receptor pathway players, tumour cells rely on the protective effect of autophagy to resist apoptosis, thereby promoting metastasis.

22.5 Autophagy Can Inhibit Tumour Invasion and Metastasis

The evidence described above suggests that autophagy can promote tumour invasion and metastasis; however, there is also evidence to support the inhibition of tumour invasion and metastasis by autophagy. The expression of Beclin1 and LC3 was downregulated in clinical tissues of tongue squamous cell carcinoma, and statistical analysis showed that this downregulation was associated with the clinical stage and degree of differentiation. The use of rapamycin to activate autophagy can effectively inhibit the proliferation, migration and invasion of tongue squamous cell carcinoma cells, while the application of the autophagy inhibitor 3-MA can promote their proliferation, migration and invasion. Similarly, studies have reported that autophagy activated by overexpression of Beclin1 can effectively inhibit the invasion and metastasis of cervical cancer cells.

Although autophagy mainly regulates biological behaviour in tumour cells, there is also evidence that autophagy can indirectly inhibit inflammation in the primary tumour. Since inflammation is an essential factor for metastasis, autophagy inhibits tumour metastasis. When tumour cells are under hypoxic and metabolic stress, autophagy is activated to protect tumour cells, inhibit the occurrence of necrosis, and thus reduce the infiltration of macrophages. Initially, this effect of autophagy

was shown to play an important role in inhibiting tumour growth; thereafter, evidence from animal models of breast cancer indicates that infiltration of macrophages at the primary tumour site is also necessary for tumour invasion and metastasis. In summary, autophagy can inhibit tumour metastasis by inhibiting macrophage infiltration. In addition, autophagy can directly mediate tumour-associated inflammatory responses by regulating the release of immunoregulatory factors, such as high-mobility group box protein 1 (HMGB1), from tumour cells. HMGB1 activates dendritic cells by binding to TLR4 on the cell surface, thereby inducing an antitumour immune response to kill tumour cells and inhibit metastasis.

Autophagy can also regulate the expression of related proteins through autophagic degradation and thus inhibit tumour invasion and metastasis. Death effector domain-containing DNA-binding protein (DEDD) inhibits EMT and is an endogenous inhibitor of tumour growth and metastasis. In clinical breast and colon cancer specimens, lower expression of DEDD was found to indicate worse patient prognosis. In vitro and in vivo experiments show that overexpression of DEDD can significantly inhibit the invasiveness of tumour cells with high metastatic properties, while silencing DEDD can enhance the invasiveness of cells with low metastatic potential. Further studies found that DEDD activates autophagy and promotes autophagic degradation of Snail and Twist by direct interaction with class III PI-3-kinase (PI3KC3)/Beclin1, thereby inhibiting EMT. Researchers found that the loss of autophagy promoted the proliferation and migration of tumour cells. Further studies revealed that P62 and TWIST1 colocalize in autophagosomes when autophagy is activated. In the absence of autophagy, P62 binds to TWIST1 and inhibits its autophagic degradation, concurrently inhibiting proteasomal degradation of TWIST1 and thereby promoting the expression of the E-cadherin gene, which in turn inhibits EMT and affects tumour invasion and metastasis, indicating that autophagy inhibits TWIST1 and EMT by regulating the protein level of P62. Researchers further identified P62 expression in human tumour tissues, including cutaneous squamous cell carcinoma, malignant melanoma and many other types of tumour tissues. Loss of autophagy leads to the accumulation of SQSTM1 and activates the transcription factor NFE2L2/NRF2 to promote the progression of liver cancer.

Autophagy can also regulate tumour invasion and metastasis through other mechanisms. CUB (C1r/C1s, urchin embryonic growth factor, BMP1) domain-containing protein 1 (CDCP1) promotes tumour metastasis through a variety of pathways, including inhibition of anoikis. Inhibition of CDCP1 by siRNA induces caspase-independent death in suspension-cultured lung cancer cells, while inhibition of CDCP1 induces activation of autophagy in lung cancer cells; however, overexpression or phosphorylation activates endogenous CDCP1 to inhibit autophagy. Further studies found that the use of autophagy inhibitors can reduce the death of suspended lung cancer cells caused by CDCP1 inhibition. This study provides evidence that autophagy promotes anoikis and inhibits the metastasis of lung cancer cells.

Knockout of GABARAPL1 inhibits the expression of LAMP1 and reduces the number of lysosomes, thereby inhibiting f autophagy; this process leads to the accumulation of damaged mitochondria, promotes the formation of mitochondria and

increases the number of mitochondria; moreover, the number of mitochondrial proteins and mitochondrial membrane potential can be observed. Mitochondrial respiration and ATP levels are elevated, providing sufficient energy for tumour cell proliferation. In addition, elevated mitochondrial ROS levels can promote increases in the mitochondrial number and glutathione levels, which further induces tumour cell tolerance to HNE protein-mediated death and ultimately promotes tumour cell invasion and metastasis. This finding provides evidence for the possible role of autophagy in inhibiting tumour progression.

It should be noted that recent studies have also confirmed that autophagy inhibits EMT. It has been observed in glioblastoma cells that starvation or rapamycin-induced autophagy attenuates cell migration and invasion; the main mechanism acts through autophagic downregulation of SNAI2 and SNAI1 expression. Silencing the Beclin 1 or ATG7 gene enhances migration and invasion, suggesting that autophagy in glioblastoma may inhibit EMT-mediated metastasis. In thyroid papillary carcinoma cells, CDH6 (cadherin 6) inhibits autophagy to promote EMT and metastasis by binding the autophagy proteins BNIP3, BNIP3L and GABARAP (GABAA receptor-associated protein). In Skov-3 ovarian cancer cells, low basal autophagy levels increase the propensity for metastasis and invasion compared to that of cells with high levels of autophagy. In addition, starvation-induced autophagy reduces invasion and metastasis and decreases the expression of the mesenchymal markers VIM, CDH2/N-cadherin and ZEB1 in cells with siRNA-mediated knockout of ATG7. In ovarian cancer cells, inhibition of autophagy enhances EMT expression by activating the ROS/HO-1 (heme oxygenase 1) pathway. These results suggest that autophagy inhibits EMT-induced invasion and metastasis by reducing TGFB1-SMAD signalling in most cases. Recently, studies have also found that autophagy reduces the invasiveness of glioblastoma by downregulating key EMT molecules such as Snail and Slug. Thus, autophagy can reduce the migration of tumour cells by indirectly inhibiting EMT (Catalano et al. 2015).

The cytoskeleton provided for tumour cells by the ECM promotes their proliferation and migration. As one of the important cell–matrix molecules, fibronectin (FN) is involved in matrix remodelling, cell adhesion and migration. There is increasing evidence that autophagy can significantly affect ECM composition and reduce tumour cell fibrosis. Dower CM et al. found that in breast cancer cells, hypoxia-induced autophagy inhibits invasion and metastasis by inhibiting tumour fibrosis. Other studies have also found that an imbalance in FN1 signalling leads to fibrosis and increased tumour stiffness, increasing the possibility of tumour cell invasion and metastasis. When EMT and fibrosis occur in tissues, invasion and metastasis are prone to occur, and these mechanisms intersect. TGFβ1 promotes the induction of EMT and fibrosis by SNAIL and TWIST through SMAD signalling. As mentioned above, autophagy negatively regulates EMT, either by P62-mediated degradation of SNAIL or by a p62-mediated decrease in TWIST stability, further confirming that autophagy inhibits FN and fibrosis, primarily by inhibiting ROS. The role of IL1β inhibition and NF-κB-induced fibronectin formation may also be dependent on the autophagic degradation of FN1 by map1s (Dower et al. 2017).

Autophagy also negatively regulates the Rho protein. Members of the Rho family of GTPases are recognized as factors regulating cell motility, and they mainly undergo migration by regulating cytoskeletal remodelling. High expression of Rho family proteins, of which RHO, RAC, and CDC42 are three representative and well-studied molecules, is associated with tumorigenesis. Activated RAC1 regulates the formation of lamellar footplates, which contribute to cell proliferation and local adhesion, and activation of CDC42 is essential for the formation of stress fibres; these processes mainly involve the regulation of cytoskeletal actin, initiation of intracellular and extracellular signalling, regulation of protein kinases and assembly of microfilaments induced by the RHO protein (Baranwal and Alahari 2011).

The earliest studies linking autophagy to the function of members of the Rho family were conducted in Drosophila, where autophagy is required for blood cell migration during wound healing. Later, more studies found that autophagy helps to stabilize RHO protein activity. Cell cycle signal termination is dependent on the degradation of many signalling proteins that regulate cell growth or promote or inhibit proliferation. Belaid A. et al. found that RHOA is a target protein whose genomic stability is maintained by autophagy—that is, autophagy, as a protective mechanism, can maintain activated RHO at a suitable level. This study found that in kidney and lung cancer cells, SQSTM1 helps to promote autophagic degradation of RHO. Deletion of the autophagy gene ATG5 increases RHO levels, leading to chromosomal instability and increased mobility. This study also found that high expression of RHO was positively correlated with the occurrence of autophagy defects in lung cancer, suggesting that the loss of autophagy may promote RHO dysregulation, leading to lung cancer progression (Belaid et al. 2013). As indicated above, autophagy can negatively regulate members of the RHO family to influence invasion and metastasis. Further mechanistic investigation found that autophagy regulates the activity of RHO dependent on GEF-H1 (guanine nucleotide exchange factor). In wild-type MEF cells, GEF-H1 directly binds p62 to be degraded by autophagy, thereby reducing the RHO levels. In addition, activated ROCK activates autophagy at starvation levels and inhibits the binding of BECN1 to BCL2L1 by phosphorylating BECN1. This finding demonstrates that RHO and ROCK1 are capable of negatively regulating BECN1-mediated autophagy activation. The expression of RHO and ROCK1 is associated with invasion and metastasis. Therefore, RHO and ROCK1-induced autophagy negatively regulate RHO and ARHGEF2, preventing chromosomal mutations and tumour progression, invasion and metastasis. These results indicate that there is a close relationship between RHO protein activity and autophagy levels and that mutual regulation between these proteins forms a complex network. More research is needed in the future to fully elucidate how Rho proteins and their effector proteins coordinate autophagy to regulate cell movement and migration (Dower et al. 2018).

22.6 Conclusions

Autophagy plays an important role in tumour development processes, including invasion and metastasis. However, in different tumour types and research models, autophagy plays different roles; it can promote the survival, invasion and metastasis of tumour cells, and there is evidence to support its completely contrasting role in inhibiting tumour development. This dichotomy poses challenging questions for autophagy and tumour research: Does autophagy directly or indirectly regulate tumour progression? By what specific mechanisms does autophagy achieve its regulatory role? Through which genes and in which cell physiological states does autophagy play a role in promoting or inhibiting tumour progression? In-depth research on autophagy has revealed its important role and specific mechanism in tumours and will help to develop individualized autophagy-targeting treatment options for tumours, improve the efficiency of cancer treatment and bring promise to patients.

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Chapter 23
Autophagy and Tumor Cell Death

Yan Cheng and Liu Cao

Abstract There exists an intricate collaboration between autophagy and other types of PCD. These processes can be activated in parallel or sequentially, and have either common or opposite objectives. Determining which interactions between them are important in the regulation of cell death. A comprehensive and in-depth study of the crosstalk between autophagy and apoptosis, necroptosis, or pyroptosis will bring breakthroughs in the treatment of many diseases, including cancer, cardiovascular disease, and neurodegenerative disorders.

Keywords Autophagy · Apoptosis · Necroptosis · Pyroptosis

Programmed cell death (PCD) is a highly regulated cellular response in multicellular organisms to control cell fate following various cellular stresses and/or extrinsic stimuli. Several types of cell death have been identified based on morphological criteria, including apoptosis, necroptosis, pyroptosis, and autophagic cell death. In brief, apoptosis is a caspase-mediated PCD with chromosome condensation, nuclear fragmentation, and membrane blebbing (Kerr et al. 1972). Necroptosis is associated with cell and organelle swelling, and promotes inflammation through leakage of cellular contents from damaged plasma membrane (Chan et al. 2015). Pyroptosis is a new form of programmed necrosis mediated by gasdermins D and E, which are cleaved by caspase family proteins to generate an N-terminal pore-forming domain, inducing the formation of pores in the cell membrane (Kovacs and Miao 2017). Autophagy is a highly conserved mechanism through which eukaryotic cells deliver cytoplasmic material to lysosomes for degradation (Noda and Inagaki 2015). Among various biological functions of autophagy, its involvement in cell death or cell survival is the one deserving particular attention. On the one hand, autophagy has been well established as an important cell survival mechanism by facilitating the degradation

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of damaged cellular components and providing the cell with energy, in response to a variety of stress conditions, including starvation, hypoxia, mitochondrial damage, and pathogen infection. On the other hand, autophagy has been implicated in the cell death process termed as type II PCD. Thus, autophagy plays a dual role in cells, acting either as a pro-survival or pro-death mechanism depending on the context. By disrupting the connection, we can either protect cells we don't want to die (as in neurodegenerative diseases) or cause diseased cells to die (as in cancer treatment). The complicated relationships between autophagy and apoptosis, necroptosis and pyroptosis, will be further discussed one by one in the next section.

23.1 Autophagy and Apoptosis

Apoptosis is essential for organism development, growth and tissue homeostasis under physiological conditions (Du Toit 2013). Apoptosis is controlled by two signaling pathways, the intrinsic and extrinsic pathways. The intrinsic pathway is initiated from intracellular stimuli such as DNA damage, which is mediated by mitochondria (Takahashi et al. 2005). The extrinsic apoptosis pathway is triggered by extracellular stimuli such as growth factor withdrawal, steroid hormones, and ligation of death receptors (Fuchs and Steller 2015; Hengartner 2000).

Autophagy and apoptosis, the two complex and interconnected cellular processes play a critical role in maintaining cellular homeostasis and dictating cell fate. They often occur in the same cell and coordinate with each other. It has been pointed out that apoptosis and autophagy may be interconnected and even simultaneously regulated by the same trigger (Liu et al. 2009).

23.1.1 The Crosstalk Between Autophagy and Apoptosis

Autophagy occurs in response to stress or damage, apoptosis is initiated in order to eliminate cells when cellular damage is too extensive. Under some circumstances, apoptosis and autophagy can exert synergetic effect while sometimes autophagy would be triggered only when apoptosis is suppressed. Autophagy can promote or suppress apoptosis, depending on the surrounding cellular environment, the different stages of human disease, or the therapeutic interventions attempted.

Autophagy is typically regarded as a pro-survival mechanism by clearing damaged components, pathogens, or aggregates and recycling intracellular macromolecules in response to a wide array of stimuli to resist lethal apoptosis. Another mechanism of inhibition of apoptosis is that autophagy can selectively remove the abundance of pro-apoptotic proteins in cells.

By contrast, autophagy also can promote apoptosis through depleting of anti-apoptotic and cell survival factors. For instance, degradation of caveolin-1 by autophagy contributes to palmitic acid-induced inflammation and apoptosis in rat

hippocampal astrocytes (Chen et al. 2018). In addition, autophagy, as type II PCD, may be a mechanism of apoptosis-independent cell death under certain circumstances. Researches demonstrated that autophagic cell death was observed in apoptosis-resistant $bax^{-/-}$, $bak^{-/-}$ mouse embryonic fibroblasts (MEFs) treatment with chemotherapeutic agents, starvation, growth factor withdrawal, and radiation (Arakawa et al. 2017). Furthermore, inhibiting autophagy by 3-MA blocked the cell death induced by chemotherapeutic agent etoposide, indicating that autophagy may promote apoptosis under certain condition.

23.1.2 The Significance of the Crosstalk Between Autophagy and Apoptosis in Human Diseases

There is a built-in system of the balance between autophagy and apoptosis contributing to the maintenance of cellular homeostasis. Any disruption of this balance might be associated with a number of pathologies and diseases, such as cancer and neurodegeneration.

23.1.2.1 Cancer

Apoptotic signaling pathway is a natural way of removing aged cells from the body that is recognized as a potential therapeutic target for malignancies. Many cancer therapies, such as radiation therapy, chemotherapy, and targeted therapies, exert their antitumor effects by inducing cell death especially apoptosis. A large of studies has reported that autophagy can be activated as a protective or survival mechanism against anticancer therapies. In accordance with this notion, pharmacological inhibition of autophagy or silencing of key autophagic genes has been shown to potentiate apoptosis signaling, thus enhancing the efficacy of anticancer therapies. For example, suppression of eEF2K-mediated autophagy aggravates ER stress and shuttles more tumor cells into the apoptotic pathway, reinforcing the cytotoxicity of curcumin and Velcade against tumor cells (Cheng et al. 2013).

By contrast, autophagy also can promote apoptosis, synergistic killing cancer cells. Blockage of autophagy by 3-MA or silencing of ATG5 prevents apoptosis in colorectal cancer cells (Won et al. 2015). In response to DNA damage, autophagy induced by p53 contributed to p53-dependent apoptosis and cancer suppression (Kenzelmann Broz et al. 2013). Phycocyanin treatment inhibits pancreatic cancer cell growth by elevating both autophagic and apoptotic cell death (Liao et al. 2016). In addition, massive autophagic response may play as an alternative death pathway without caspase activation in cancer cells that are resistant to apoptosis-inducing therapies, including radiation, chemotherapy, and growth factor antagonists, to kill tumor cells by autophagic cell death. For example, autophagy functions as cell death

mechanism in Bax- and PUMA-deficient colon cancer cells that fail to induce apoptosis in response to 5-FU (Peng et al. 2010). Therefore, induction of autophagic death may be a viable therapeutic strategy in apoptosis-resistant tumor.

23.1.2.2 Neurodegenerative Disorders

Neurodegenerative disorders are becoming increasingly prevalent because of a larger percentage of members living to an older age. Autophagy, one elimination pathway to degradation long-lived protein in cells, controls the quality of cellular components and maintains cell homeostasis. Thus, defect in autophagy plays a pivotal role in the etiology and/or progress of neurodegenerative diseases. The relationship between autophagy and apoptosis is involved in several neurodegenerative diseases, including Parkinson's disease (PD), Huntington's disease (HD), and Alzheimer's disease (AD) (Ghavami et al. 2014).

Autophagy is now widely considered as a vital homeostatic and cytoprotective mechanism in healthy neuronal cells. This protective effect of autophagy is not simply a function of degradation of pathogenic proteins and damaged organelles but appears to be related to antiapoptotic effects on neurons (Xue et al. 2016). Autophagy can decrease the amount of mitochondria, resulting in less release of toxic molecules like cytochrome *c* from mitochondria in response to pro-apoptotic stimuli (Ghavami et al. 2014). Piperlongumine (PLG), an alkaloid extracted from *Piper longum* L., exerted the protective effects in rotenone-induced PD cell and mouse models via inhibition of apoptosis and induction of autophagy through enhancement of Bcl-2 phosphorylation at Ser70 (Liu et al. 2018).

However, the connection between autophagy and apoptosis in certain context has opposite effect. It has been reported that angiotensin II (Ang II), the main component of renin–angiotensin system, triggered apoptosis via activation of autophagy in the pathogenesis of PD. Thus, insight into the balance between apoptosis and autophagy in neurodegenerative disorders is critical for us to find a promising treatment strategy.

23.1.3 Regulatory Molecules Between Autophagy and Apoptosis

Autophagy and apoptosis are observed coexist in cells under certain circumstance as the result of shared common upstream signals and stimuli. The regulators modulating the crosstalk between autophagy and apoptosis are multifaceted and complex depending on circumstance. A number of proteins (e.g., Bcl-2, p53, caspase) and common pathways (e.g., PI3K/Akt/mTOR, ERK) have been reported to be involved in the regulation of two fundamental processes (Fig. 23.1).

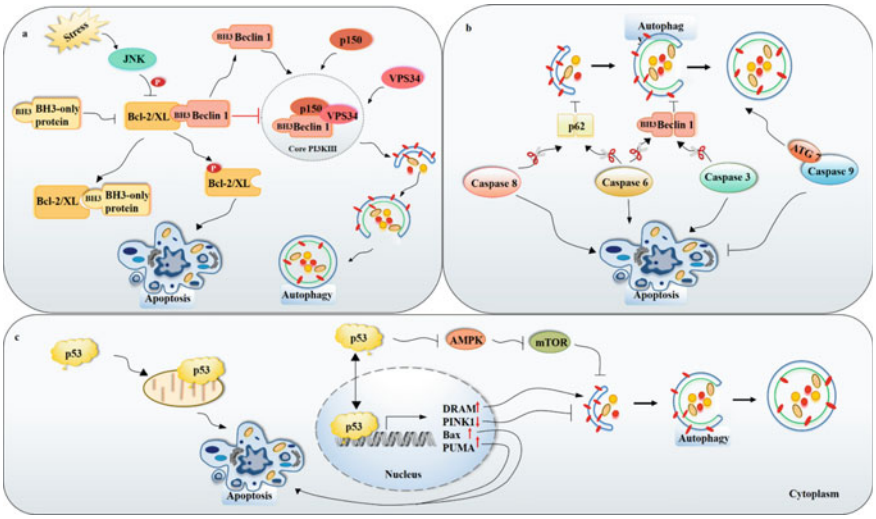

Fig. 23.1 The regulators involved in autophagy and apoptosis. **a** The role of Bcl-2 in modulation autophagy and apoptosis. **b** The effects of caspase in regulating autophagy and apoptosis. **c** Regulation of autophagy and apoptosis by tumor suppressor p53

23.1.3.1 Bcl-2 Family Proteins

Bcl-2 family proteins are the major regulators of intrinsic pathways of apoptosis though regulating MOMP. It is now well documented that these proteins have additional roles related to autophagy by interacting with some autophagy-related proteins (Gross and Katz 2017). Antiapoptosis Bcl-2 family members such as Bcl-2, Bcl-xl enable to interaction with the BH3 domain of Beclin 1, the key regulator of autophagy initiation, to inhibit autophagy (Kang et al. 2011). Posttranslational modifications of Beclin 1 or BCL-2 contribute to their interaction and dissociation, thus shifting the balance between autophagy and apoptosis. Phosphorylation of Beclin 1 at thr108 by mammalian Ste20-like kinase 1 (Mst1) enhances the interaction between Beclin1 and Bcl-2/Bcl-xL and suppressed autophagy (Maejima et al. 2013). In contrast, phosphorylation of Beclin 1 at thr119 by death-associated protein kinase 1 (DAPK1) resulted in the dissociation of Beclin 1 from Bcl-XL and the induction of autophagy (Zalckvar et al. 2009). Mutations in Bcl-2 phosphorylation sites including Thr69Ala, Ser70Ala, and Ser84Ala blocked the separation of the Bcl-2/Beclin1 complex, thus inhibiting the activation of autophagy. Active JNK1 phosphorylates Bcl-2 in response to stress or BH3 (Bcl-2 homology 3)-only proteins and BH3 mimetics, leads to the dissociation of Beclin 1 from Bcl-2/Bcl-XL, which actives autophagy by promoting the formation of Beclin 1–Vps34–p150 complex and promotes apoptosis by inactivating Bcl-2 (He et al. 2012). In addition, cleavage of Beclin1 by caspase3 disrupts the complex formation and promotes the switch between autophagy and apoptosis (He et al. 2012). Pharmacological BH3 mimetics are promising agents for disease treatment to

induce autophagy or apoptosis by blocking the interaction between Bcl-2 or Bcl-XL and Beclin1 (Malik et al. 2011).

23.1.3.2 Caspases

Caspases are widely known as the critical mediators of the apoptosis. It has been shown that caspases participate in the regulation of autophagy by cleavage of several autophagy-related proteins, including p62, Beclin1, VPS34, ATG3, ATG4D, and AMBRA1 (Booth et al. 2014). Different caspases can recognize and interact with different autophagy effectors to exert specific roles. Studies have shown that caspase-6 and -8 can inhibit autophagy through cleavage of p62 and Beclin1. As an initiator caspase, caspase-9 can inhibit autophagy via cleaving and inactivating ATG5 and Beclin1 (You et al. 2013). In contrast, caspase-9 also promotes autophagy interaction with ATG7. Cleavage of Beclin-1 at TDVD133 and DQLD149 by caspase-3 yields fragments lacking pro-autophagic capacity (Wirawan et al. 2010). Therefore, activated caspases not only perform apoptosis, but also hinder autophagy by cleavage of autophagy-related proteins.

23.1.3.3 The p53 Protein

The transcription factor p53 is a principal tumor suppressor that functions as an important cellular stress sensor by regulating cell cycle arrest, apoptosis, and autophagy in response to various stressors including DNA damage, ischemia reperfusion and nutrient stress. Activation of p53 in response to a death stimulus promotes the transcription of PUMA, AMPK, and Bax, leading to activate intrinsic mitochondrial apoptotic pathway. The function of p53 in regulation of autophagy is complex and dependent upon subcellular localization (Van Nostrand et al. 2015). The cytosolic p53 represses autophagy by blocking autophagosome formation via AMPK/mTOR pathway. Nevertheless, nuclear translocation of p53 promotes the induction of autophagy (Tang et al. 2015). Recently, it has been reported that nuclear p53 could repress mitophagy by repressing PINK1 gene transcription (Goiran et al. 2018). So, the regulatory role and mechanism of nuclear p53 on autophagy remains to be further explored.

23.2 Autophagy and Necroptosis

Necrosis has been seen as an accidental and uncontrolled form of cell death for many years. However, with the evolution of genetic, biochemical evidence and the discovery of specific chemical inhibitors of necrosis, necrosis has been discovered to be also a programmed and regulated cell death process, including necroptosis, mitochondrial permeability transition (MPT)-driven regulated necrosis, parthanatos,

Table 23.1 Crosstalk between autophagy and necroptosis

Interactions	Examples
Autophagy promotes necroptosis	• Inhibition of BMI1 could induce autophagy-mediated necroptosis in OvCa cells • HSP70 downregulates cardiomyocyte necroptosis through suppressing autophagy during myocardial IR • Chalcone-24 (Chal-24), a novel chalcone derivative, exerts antitumor effects through autophagy-mediated necroptosis
Autophagy suppresses necroptosis	• zVAD, a pan-caspase inhibitors that is capable of preventing apoptosis, triggers necroptosis. Suppresses of autophagy via inhibition of lysosomal function contributes to zVAD-induced necrotic cell death • Suppression of autophagic flux contributes to RIP1–RIP3 interaction and necroptosis of cardiomyocytes • Shikonin, a natural naphthoquinone pigment, can induce necroptosis and autophagy in NSCLC cells, and the inhibition of shikonin-induced autophagy enhances necroptosis
Autophagy has no effects on necroptosis	• TNFα administration caused necroptosis and autophagy; however, autophagy inhibitor 3-methyladenine (3-MA) did not affect RIP1 expression

and ferroptosis. Among various forms of necrotic cell death, necroptosis is the very important form, termed as type III PCD (Galluzzi et al. 2017).

The interaction between autophagy and necroptosis is rather complex, depending on the specific circumstances. Autophagy is able to either promote or suppress necroptosis or is not associated with necroptosis (Table 23.1).

The ability of autophagy to suppress necrotic cell death is considered to be one of the most important pro-survival functions of autophagy. For example, autophagy protein ATG16L1 prevents necroptosis in the intestinal epithelium. Suppression of autophagy contributes to zVAD-induced necrotic cell death, indicating the pro-survival function of autophagy in zVAD-induced necroptosis (Wu et al. 2009). In addition, suppression of autophagic flux contributes to RIP1–RIP3 interaction and necroptosis of cardiomyocytes (Ogasawara et al. 2017). Shikonin, a natural naphthoquinone pigment purified from Lithospermumerythrorhizon, can induce necroptosis and autophagy in NSCLC cells. Furthermore, the inhibition of shikonin-induced autophagy enhanced necroptosis (Kim et al. 2017).

However, several studies showed that autophagy is able to promote necroptosis. In children with acute lymphoblastic leukemia (ALL), combination of rapamycin with the glucocorticoid dexamethasone trigger autophagy-dependent cell death, with characteristic features of necroptosis, suggesting autophagy promotes necroptosis in this particular system (Bonapace et al. 2010). Inhibition of BMI1 by either siRNA or a

small molecule inhibitor significantly inhibits clonal growth in OvCa cells by inducing autophagy-mediated necroptosis (Dey et al. 2016). The induction of autophagy leads to the activation of necroptosis in a p62-dependent manner in prostate cancer cells in response to sorafenib, and inhibition of necroptosis renders the cells resistant to sorafenib. Chalcone-24 (Chal-24), a novel chalcone derivative, exerts antitumor effects through inducing autophagy-mediated necroptosis (He et al. 2014). HSP70 is regarded as a cardiac protective molecule and could be upregulated to inhibit cardiomyocyte apoptosis after MIR (Peng et al. 2010). It has been found that HSP70 downregulates cardiomyocyte necroptosis through suppressing autophagy during myocardial IR, revealing the novel protective mechanism of HSP70.

TNFα administration caused mitochondrial dysfunction and reactive oxygen species (ROS) production, which led to necroptosis and autophagy in murine fibrosarcoma L929 cells. Nec-1 repressed mitochondrial dysfunction and ROS production; however, autophagy inhibitor 3-MA did not affect mitochondrial dysfunction and ROS production. These results indicate autophagy has no effects on necroptosis under the particular circumstances.

23.3 Autophagy and Pyroptosis

Pyroptosis was initially thought to be caspase-1-dependent monocyte death (Bergsbaken et al. 2009). Subsequently, it was found that caspase-11/4/5 like caspase-1, could also cleave gasdermin D (GSDMD) to generate an N-terminal pore-forming domain, which oligomerizes to form large pores in the plasma membrane, driving swelling, and membrane rupture (Liu et al. 2016). Thus, the cell pyroptosis was redefined as a programmed necrosis mediated by gasdermin. In 2017, there was a new breakthrough in pyroptosis, revealing that gasdermin E (DFNA5), another gasdermin family protein, can be cleaved by activated caspase-3 to generate a DFNA5-N fragment that targets the plasma membrane to induce pyroptosis (Wang et al. 2017).

Although autophagy has been described as a death mechanism, the consensus is that autophagy can also play a primarily protective role in several death stimulations including pyroptosis. At present, there are few reports about the relationship between autophagy and pyroptosis.

NLRs are one of the important ways of inducing pyroptosis by triggering activation of caspase-1 and processing the release of the inflammatory cytokines IL-18 and IL-1β (Bergsbaken et al. 2009). At present, reports on the relationship between autophagy and pyroptosis are almost all related to this pathway. For instance, inhibition of autophagy led to activation of the NLRP3 inflammasome and caspase-1, increased secretion of IL-1β and IL-18 in LPS-stimulated macrophages and in sepsis model (Saitoh et al. 2008). In contrast, the induction of autophagy using rapamycin reduced LPS-induced elevation of serum IL-1β (Harris et al. 2011).

It has been reported that several intracellular bacterial pathogens that trigger pyroptosis also induce autophagy. For example, caspase1-dependent cell death occurred more frequently when autophagy was dampened by either 3-MA or an

inhibitor of the ATG4 protease in L. pneumophila-infected macrophages (Byrne et al. 2013). Inhibition of autophagy promoted pyroptosis in Shigella-infected macrophages (Suzuki et al. 2007). These reports suggest that autophagy protects infected macrophages from pyroptosis. In addition, autophagy was stimulated and protected pneumococcus-infected microglia from pyroptosis. An important role of autophagy in host defense against P. aeruginosa-induced sepsis in mice was also recently disclosed. In the referred work, ATG7 was involved in inflammasome activation in pseudomonas aeruginosa abdominal infection, and loss of ATG7 led to an increase in IL-1β and pyroptosis, consistent with enhanced inflammasome activation (Pu et al. 2017).

Overall, it is reasonable to conclude that autophagy inhibition results in pyroptosis activation. Indeed, autophagy may function as a negative regulatory mechanism for pyroptosis, thus providing a checkpoint to promote the development of pyroptosis. It seems to be a promising approach to promote the pyroptosis process by inhibiting autophagy or targeting directly specific NLRs to reduce their activity. But the interaction between pyroptosis and autophagy is complex and easily affected by various factors. Therefore, it is difficult to achieve this goal. It is worth mentioning that the specific regulatory mechanisms how autophagy antagonizes pyroptosis are not clear at present. Thus, a major open question in the field is to determine how autophagy has generally inhibitory effects on pyroptosis.

The relationship between autophagy and cell death is controversial and highly context-dependent. They coordinate with one another and modulate the survival and death of cells together in response to special cellular stress. Any disruption of this balance might be associated with a number of diseases. Here, we summarized the recent advances of the interplay between autophagy and PCD in controlling cell survival and death. The key regulators and the protein–protein interactions between autophagic and other types of cell death have been discussed. Recently, studies mainly focus on the complex relationship between autophagy and apoptosis. While the functions and the relative molecular mechanisms of autophagy in regulating necroptosis and pyroptosis is largely unknown. Determining which interactions between them are important in regulation of cell death. A comprehensive and in-depth study of the crosstalk between autophagy and apoptosis, necroptosis, or pyroptosis will bring breakthroughs in treatment of many diseases, including cancer, cardiovascular disease, and neurodegenerative disorders. By disrupting the connection, we can either protect cells we don't want to die (as in neurodegenerative diseases) or cause diseased cells to die (as in cancer treatment).

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Chapter 24
Autophagy and Tumour Chemotherapy

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Abstract Chemotherapy is an important means of treating malignant tumours. The main role of chemotherapy drugs is to induce cell death. However, the apoptotic pathways of many tumour cells are often severely impaired, leading to failure of chemotherapy-induced apoptosis. With the in-depth study of autophagy in recent years, this process has been found to play an important role in the chemoresistance of tumours. Autophagy may have different effects on tumour cells depending on the specific environment. In addition, tumour stem cells and the tumour microenvironment are closely related to tumour recurrence and metastasis. It is also important to study the role of autophagy in tumour stem cells and the microenvironment to investigate chemotherapy resistance.

Keywords Chemoresistance · Autophagy · Tumour stem cells · Tumour microenvironment

24.1 Introduction

Chemotherapy is an important method for treating malignant tumours. In the 1940s, Gilman discovered that nitrogen mustard could treat lymphoma, which was the precursor of tumour chemotherapy. Then, Faber used the folate analogue methotrexate (MTX) to treat acute lymphoblastic leukaemia and achieved remission. In the 1970s, cisplatin and adriamycin were used in the clinic, and some patients were cured. The application of taxanes and camptothecin in the 1990s further improved the 5-year survival rate of some patients. After entering the twenty-first century, the types of chemotherapy drugs increased, and chemotherapy methods are improved day by day, with chemotherapy drugs playing a very important role in the treatment of tumours.

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The development of chemotherapeutic drugs has experienced different stages, from palliative treatment to radical treatment, and some breakthroughs have been made in adjuvant chemotherapy, chemotherapeutic adjuvant drugs, targeted therapy and cellular immunotherapy. At present, chemotherapy has made remarkable progress in the treatment of leukaemia, malignant lymphoma, head and neck tumours, digestive system and respiratory system tumours and others. Some tumours can be completely relieved or even cured through chemotherapy. The commonly used chemotherapeutic drugs act on different cell growth processes according to their different mechanisms. Drugs such as MTX and 5-fluorouracil (5-FU) interfere with DNA or RNA biosynthesis; nitrogen mustard, cyclophosphamide, platinum and other drugs directly destroy the structure and function of DNA. Drugs such as actinomycin and pirarubicin insert into DNA to interfere with RNA transcription. Drugs such as vincristine and etoposide interfere with protein synthesis. Other chemotherapeutic agents include corticosteroids, oestrogen, tamoxifen and other hormone drugs.

The update and extensive use of chemotherapy drugs have significantly improved the quality of life of tumour patients and extended the survival time of patients, but tolerance of chemotherapy drugs is still the bottleneck restricting their efficacy. Most patients will have different degrees of drug resistance after using chemotherapy drugs for a period of time, and the possible mechanisms include: ① cell apoptosis tolerance, ② enhanced drug efflux ability, ③ improvement of DNA damage repair function and ④ others such as ischaemia, hypoxia and other microenvironment factors.

The main function of chemotherapy drugs is to induce cell death. Apoptosis is one of the most studied cell death modes in recent years. The apoptosis signalling pathways mainly occur through mitochondria-dependent endogenous pathways, such as the Fas/FasL or TNF/TNFR pathway, and death receptor-dependent exogenous pathways, activating caspase 8 or 9 and the downstream apoptotic key molecule caspase 3, to induce apoptosis. The apoptosis rate of monocytes extracted from the peripheral blood of leukaemia patients was significantly increased under the action of chemotherapy drugs (cytarabine, mitoxantrone, etoposide, etc.). Similar results were found in solid tumours. Yusu et al. proposed that the mechanism of chemotherapy-induced apoptosis can be divided into three phases: the injury generation phase, signal transduction phase and apoptosis induction phase. In the apoptosis-induced phase, depending on the strength of the injury signal, the cell will enter the blocking process for damage repair or enter the apoptosis pathway. When apoptosis is blocked, damaged cells cannot enter the apoptosis pathway, thus showing decreased drug sensitivity or drug resistance. In fact, the apoptotic pathways of many tumour cells are often severely impaired, leading to the failure of chemotherapeutic drug-induced apoptosis.

With the in-depth research on autophagy in recent years, its role in chemotherapy resistance has attracted much attention. Different from apoptosis, autophagy is a process of cell digestion, mainly through lysosomal degradation of damaged organelles and proteins. It is very important to maintain cell homeostasis and adapt to the stimuli of a changing environment. Studies have indicated that chemotherapy drugs acting on different tumour cells can induce the production of autophagy. There are two different results of the autophagy process in tumour cells induced

by chemotherapy drugs. One is that autophagy, as the self-protection mechanism of tumour cells under stress, inhibits the chronic inflammatory reaction by clearing damaged organelles to avoid the damage caused by chemotherapy and induce the development of drug tolerance. The other is that chemotherapy drugs directly or indirectly initiate autophagy-induced non-apoptotic death (also known as autophagy death) to exert tumour inhibition and promote the sensitivity of tumour cells to chemotherapy. Therefore, autophagy may play a dual regulatory role in the processes involved in tumour chemotherapy, and autophagy may have different effects on tumour cells according to the specific environment. In addition, tumour stem cells and the tumour microenvironment are closely related to tumour recurrence and metastasis, so it is of great significance to pay attention to the role of autophagy in tumour stem cells and the microenvironment for the study of chemotherapy resistance. Because of the close connection between autophagy and tumour chemotherapy resistance, the study of this new field and exploration of the specific mechanisms and intervention methods between these processes may improve the sensitivity of chemotherapy to a certain extent, thus providing new ideas for clinical treatment.

24.2 Autophagy and Chemosensitivity

24.2.1 Autophagy Activation Promotes Chemotherapy Resistance

Chemotherapy resistance has always been the main bottleneck mediating the effects of chemotherapy. According to its source, chemotherapy resistance can be divided into endogenous resistance and acquired resistance. Endogenous chemotherapy resistance already exists before individuals undergo chemotherapy, such as that occurring in hepatocellular carcinoma and melanocarcinoma. Acquired chemotherapy resistance is a kind of drug resistance formed after individuals undergo chemotherapy, which can be induced by a single chemotherapeutic drug or the interaction of several chemotherapeutic drugs. Many tumours are sensitive to chemotherapeutic drugs at the beginning but become insensitive to similar chemotherapeutic drugs after a period of treatment. The factors affecting endogenous chemotherapy resistance include drug absorption and metabolism, DNA damage repair function and apoptosis ability. The factors affecting acquired chemotherapy resistance include hypoxia, nutritional deficiency, tumour heterogeneity, gene or epigenetic changes induced by the microenvironment and autophagy activation. Therefore, many factors including autophagy can affect the response of tumour cells to chemotherapeutic drugs, resulting in varying degrees of chemotherapy resistance.

Autophagy is a mechanism unique to eukaryotic cells that relies on lysosomal self-digestion and intracellular recycling. It is an important way for cells to remove damaged organelles or proteins, maintain homeostasis of the intracellular environment, and provide raw materials to maintain the critical mechanisms of cells in

nutrient deficiency. Some of the cytoplasm and organelles are isolated by the bilayer membrane and then transported to the lysosome for degradation via the process of autophagy. Autophagy can be divided into three types, macroautophagy, microautophagy, and chaperone-mediated autophagy, depending on the way intracellular substances are transported to lysosomes. The process that is usually called autophagy is macroautophagy. As a feature specific to housekeeping genes, autophagy plays an important protective role when cells are in an unfavourable environment. Studies have confirmed that autophagy can promote the survival of tumour cells. Tumour cells are in a state of ischaemia and are nutrient-deprived in the late stage of tumorigenesis. Autophagy can help them adapt to the adverse tumour microenvironment and promote their survival. Autophagy can also protect tumour cells from apoptosis and maintain malignant proliferation by eliminating intracellular harmful substances and providing substrates and energy for the repair of damaged DNA during chemotherapy. Studies demonstrated that loss of the selective autophagy receptor p62 impaired expansion and the colony-forming ability of leukaemia cells and prolonged the latency of leukaemia development in mice. High p62 expression was associated with poor prognosis in human acute myeloid leukaemia (AML). The results highlight the prominent role of selective autophagy in leukaemia progression (Nguyen et al. 2018).

Studies of chemotherapy resistance in clinics found that autophagy can promote tumour cells to adapt to changes in the tumour microenvironment induced by chemotherapy as a protective mechanism, which is an important reason for mediating chemotherapy resistance. Autophagy inhibitors may represent a breakthrough to improve the effects of chemotherapy given that autophagy promotes the chemotherapy resistance of tumour cells. Chemotherapy drugs not only kill cancer cells directly but also drive immune responses to target killing of the remaining cancer cells. The T cell immune response is independent of the autophagy activity of tumour cells in immune-competent mouse models of melanoma and mammary cancer. Antitumour adaptive immunity is not adversely impaired by autophagy inhibition and can enhance the tumour-killing effect of certain types of chemotherapy drugs. Chloroquine (CQ) and hydroxychloroquine (HCQ) are two autophagy inhibitors approved by the Food and Drug Administration (FDA) of the United States. They function mainly by blocking the fusion and degradation of autophagosomes. Many clinical trials have been registered in the clinicaltrials.gov database for treating multiple types of tumour patients with CQ or HCQ. CQ or HCQ is commonly used in combination with other antitumour drugs in these clinical trials (Verbaanderd et al. 2017). The safety and antitumour activity of CQ and HCQ in tumours were initially confirmed based on published clinical trial data. HCQ has a wider range of safe doses than CQ. Ferroquine (FQ) is a next-generation antimalarial drug that can effectively inhibit autophagy by disrupting the function of lysosomes in vivo. Studies have shown that FQ can inhibit the growth of advanced solid tumours, especially under nutrient deficiency and a hypoxic microenvironment. FQ can enhance the antitumour activity of several chemotherapeutic drugs, indicating its potential application as an adjuvant to existing antitumour therapy.

The effects of autophagy on chemotherapy resistance in several common tumours are described below.

24.2.1.1 Digestive System Tumours

Hepatocellular carcinoma is not sensitive to chemotherapy and has strong endogenous resistance. It has been reported that sorafenib-induced autophagy is an important mechanism for the induction of chemotherapy resistance. Autophagy inhibitors combined with chemotherapy drugs or molecular targeted drugs are considered to be an effective strategy for the future treatment of liver cancer. Combined with sorafenib alone, the autophagy inhibitor CQ enhanced endoplasmic reticulum-induced cell death. Autophagy inhibitors combined with bevacizumab significantly inhibited the growth of liver cancer cells. Oxaliplatin can significantly increase the level of autophagy in liver cancer cell lines, and inhibition of autophagy can enhance the death of liver cancer cells induced by oxaliplatin. Transarterial chemoembolization (TACE) can play a dual role in embolization and chemotherapy. Studies confirmed that compared with TACE alone, the antitumour effect of a CQ combination group was significantly enhanced, which suggests that autophagy activation may promote the chemotherapy resistance of liver cancer cells; CQ may be used as an adjuvant to improve the effect of TACE in the treatment of liver cancer.

5-Fluorouracil (5-FU) combined with platinum is the first-line drug for clinical colorectal cancer chemotherapy. It has been reported that the autophagy inhibitor CQ or 3-methyladenine (3-MA) in vitro and in vivo can significantly enhance the antitumour effect of 5-FU. Studies of HCQ combined with bevacizumab/FOLFOX are currently underway. It has recently been found that mitogen-activated protein kinase 14 (MAPK 14)/P38α is involved in the resistance of colon cancer cells to 5-FU and irinotecan chemotherapeutic drugs by promoting autophagy. In addition, in HCT116 colon cancer cells, apigenin can induce apoptosis and autophagy, while autophagy plays a protective role in apigenin-induced tumour cell apoptosis; the autophagy inhibitor 3-MA combined with apigenin may improve the antitumour effect of the latter single drug on colon cancer.

In a phase I/II clinical trial, 35 patients with pancreatic cancer were treated with HCQ (1200 mg/day) and gemcitabine before surgery. The results showed that 19 patients had a decrease in CA19-9 after treatment, and 29 patients underwent surgical resection. The R0 resection rate was 77%, and the median overall survival was 34.8 months. A phase II clinical trial examined the safety and efficacy of twice-weekly 400 or 600 mg HCQ monotherapy in 20 patients with previously treated metastatic pancreatic cancer, but the 2-month progression-free survival rate was only 10%, with no significant difference between groups.

24.2.1.2 Breast Tumours

Endocrine therapy is a common treatment for oestrogen receptor (ER)-positive breast cancer patients. The oestrogen antagonist tamoxifen is widely used in patients with ER-positive breast cancer, but patients are susceptible to drug resistance. Changes in autophagy/autophagy function are important mechanisms of tamoxifen resistance, and inhibition of autophagy can significantly enhance the sensitivity of tamoxifen to endocrine therapy in breast cancer. Studies have found that metastasis-associated protein 1 (MTA1) induces adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK) activation, and subsequent autophagy may contribute to breast cancer development of tamoxifen resistance (Lee et al. 2018). Trastuzumab is an anti-HER2 monoclonal antibody that specifically binds to the HER2 gene and blocks the HER2 expression pathway. It is suitable for HER2-positive breast cancer patients, but the single-targeted drug treatment is not effective. The autophagy inhibitor 3-MA combined with trastuzumab increased the antitumour effect of HER2-positive breast cancer. Lapatinib is a novel HER2/EGFR tyrosine kinase inhibitor suitable for HER2-positive breast cancer patients but is susceptible to acquired resistance. Inhibition of autophagy reduces the proliferation, DNA synthesis and colony-forming ability of lapatinib-resistant cells. For triple-negative breast cancer, inhibition of autophagy also showed the same antitumour effect. Nanoparticle-mediated autophagy inhibition can promote the killing effect of chemotherapy drugs on breast cancer cells. A number of phase I and II clinical trials have found that autophagy inhibitors (CQ, HCQ) alone/in combination with endocrine therapy, chemotherapy and targeted therapy can improve the therapeutic effect against breast cancer (Verbaanderd et al. 2017). The combination of an autophagy inhibitor and chemotherapeutic drugs may show good therapeutic prospects.

24.2.1.3 Respiratory Tumours

Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) have been widely used in patients with advanced lung adenocarcinoma with activated EGFR mutations. However, due to endogenous or acquired resistance, the efficacy of these drugs is limited. Gefitinib/erlotinib treatment in lung cancer cells activates autophagy and promotes acquired resistance to EGFR-TKIs. Pemetrexed is a multi-target antifolate used to treat malignant mesothelioma and non-small-cell lung cancer (NSCLC) and has been shown to stimulate autophagy. Inhibition of autophagy enhanced pemetrexed and simvastatin-induced malignant mesothelioma and apoptotic cell death in NSCLC cells. In patients with advanced lung adenocarcinoma, 11 autophagy core genes have multiple functional genetic polymorphisms, which are significantly associated with patient survival and prognosis and are associated with endogenous or acquired resistance to gefitinib (Yuan et al. 2017). CQ has been used to improve the sensitivity of lung cancer chemotherapy, not only to enhance the cytotoxicity of topotecan (TPT) but also to enhance the induction of apoptosis by the PI3K/mTOR inhibitor NVP-BEZ235, inhibiting the colony formation of tumour cells and the

growth of metastases. Phase I clinical trials in 27 patients with advanced NSCLC showed that the combination of HCQ and erlotinib was safely used with a daily dose of HCQ 1000 mg and erlotinib 150 mg. Cepharanthine (CEP) is an alkaloid extracted from the genus Asteraceae and is a novel autophagy inhibitor. Dacomitinib (DAC) is a second-generation EGFR inhibitor. In phase III clinical trials of NSCLC treatment, DAC-induced protective autophagy reduced its anticancer effect, while CEP combined with DAC increased the anticancer effect of DAC in xenograft mice (Tang et al. 2018).

24.2.1.4 Urinary Tumours

Autophagy as a survival mechanism can promote the resistance of prostate cancer and kidney cancer. Recent studies have shown that activation of autophagy protects endoplasmic reticulum-induced cell death, while inhibition of autophagy can inhibit the growth of the prostate cancer cell line PC-3 in vivo. In prostate cancer cells, inhibition of autophagy with HCQ or beclin-1/Atg5 (autophagy-associated gene 5) small interfering RNA enhances ABT-737 cytotoxicity and ursolic acid-induced apoptosis; for patients with metastatic renal cell carcinoma, although a high dose of interleukin-2 (IL-2) is effective, it is accompanied by obvious side effects, which may result in systemic autophagy syndrome. In prostate cancer, autophagy-associated gene 7 (Atg7) is capable of promoting OCT4 transcription, thereby enhancing the resistance to androgen blockade therapy (ADT) and maintaining the dry characteristics of cancer stem cells. Liang et al. found that compared with IL-2 monotherapy, IL-2 combined with CQ can significantly increase its antitumour activity while reducing the drug side effects, providing a new idea for the treatment of kidney cancer.

24.2.1.5 Nervous System Tumours

The treatment of glioma mainly includes chemotherapy and targeted therapy, and chemotherapy resistance is an important cause of treatment failure. Temozolomide (TM) is a widely used chemotherapeutic drug for the treatment of gliomas. TM has a definite effect on glioblastoma patients who are resistant to pro-apoptotic chemotherapy. Studies have shown that therapeutic doses of temozolomide (TMZ) can induce autophagy, and the autophagy inhibitor bafilomycin A1 can enhance the activation of caspase 3 and induce apoptosis. The occurrence of sputum suggests that autophagy is a mechanism by which glioma cells escape death. Bevacizumab is a human vascular endothelial growth factor (VEGF) inhibitor. Monotherapy for glioma treatment is prone to drug resistance; combined use with the autophagy inhibitor CQ can improve the antitumour effect of bevacizumab. A randomized, double-blind trial investigated the effects of combined CQ on conventional treatment of gliomas. The median overall survival of the patients in the CQ-treated group was 24 months, compared with 11 months in the placebo group. This result suggests that inhibition of autophagy in

glioblastoma may be an important direction for improving the effect of targeted therapy. In the treatment of pituitary tumours, the combination of the dopamine receptor agonist cabergoline (CAB) and CQ can improve the clinical effectiveness of treating pituitary tumours (Lin et al. 2017). A phase I/II clinical trial examined the efficacy and safety of HCQ in combination with radiation therapy and temozolomide for glioma. The results indicate that the maximum tolerated dose of HCQ per day is 600 mg.

24.2.1.6 Haematological Tumours

The tyrosine kinase inhibitor imatinib (TKI) is an important treatment for patients with chronic myeloid leukaemia (CML). The chemotherapy resistance of CML stem cells and the resistance induced by TKI-induced autophagy are key factors contributing to leukaemia recurrence and metastasis. Studies have shown that inhibition of autophagy can restore the sensitivity of CML stem cells to TKI, promote tumour cell death, and enhance the efficacy of chemotherapy drugs. In addition, in recent years, scientists have found that cobalt chloride (which induces hypoxia) can increase the differentiation of acute promyelocytic and other acute myeloid leukaemia cells while inhibiting autophagy can promote leukaemia cell differentiation or apoptosis. Yan Z. W., et al. found that CQ combined with cobalt chloride can promote the differentiation of leukaemia cells; for imatinib-resistant slow granules, the inhibition of autophagy combined with histone deacetylase (HDAC) has become a new treatment for refractory chronic particles. Similarly, inhibition of autophagy can enhance the apoptosis of the leukaemia cell line K562 induced by daunorubicin. A phase II clinical trial examined CQ in combination with the proteasome inhibitors bortezomib and cyclophosphamide for the treatment of patients with relapsed and refractory multiple myeloma. Studies have shown that the addition of bortezomib and cyclophosphamide to CQ is effective in overcoming proteasome inhibitor resistance in most severely pretreated patients with acceptable toxicity characteristics. According to a phase I clinical trial of HCQ and bortezomib in 25 patients with relapsed or refractory myeloma, the dose of 600 mg of HCQ twice daily is safe and tolerable in combination with the standard dose of bortezomib and can improve the treatment of myeloma.

24.2.1.7 Sarcoma and Other Solid Tumours

Rapamycin and HCQ dual autophagy inhibitor treatment studies were performed in 10 patients with sarcoma who failed first-line treatment. Studies have shown that non-proliferative glycolysis occurs mainly in the cancer-associated fibroblast (CAF) region, and dual autophagy inhibitor treatment significantly reduces glycolysis activity. Phase I clinical trials of HCQ and temsirolimus (mTOR inhibitors) in patients with advanced solid tumours and melanoma have shown that the combination of mTOR inhibitors and HCQ is safe and tolerable, regulates autophagy in patients and

induces a significant antitumour effect. Vorinostat is an HDAC inhibitor. A phase I clinical trial examined the safety and initial efficacy of HCQ and vorinostat in the treatment of 27 patients with advanced solid tumours. The results showed that one patient with renal cell carcinoma was confirmed to have a persistent partial response, and two patients with colorectal cancer were able to prolong disease stability.

In summary, autophagy as a chemoresistance mechanism can promote the survival of tumour cells and become an important factor in the induction of chemotherapy resistance. Inhibition of autophagy may be an effective way to reduce chemotherapy resistance, and autophagy inhibitors combined with chemotherapy drugs may become potential strategies to improve antitumour efficacy.

24.2.2 The Mechanisms of Autophagy Regulating Tumour Cells During Chemotherapy

24.2.2.1 PI3K/AKT/mTOR and MEK/ERK

PI3K/AKT/mTOR is an important pathway regulating tumour cell growth and autophagy and has been shown to be involved in the chemotherapy resistance of tumour cells. Doxorubicin (DOX) is an effective anticancer drug, but its clinical application is limited due to its cardiotoxicity. Li et al. found that blocking PI3Kγ in a breast cancer model prevented doxorubicin-induced cardiotoxicity. At the same time, its antitumour activity is elevated by enhancing anticancer immunity. PI3Kγ inhibitors act by enhancing the autophagy clearance of mitochondria by doxorubicin. Neri LM et al. reported that doxorubicin combined with AKT inhibitors in B-progenitor lymphoblastic leukaemia can promote apoptosis. Westhoff MA et al. found that doxorubicin-induced apoptosis was significantly increased in neuroblastoma with the PI3K/mTOR inhibitor NVP-BEZ235. Li H. et al. also confirmed that NVP-BEZ235 can induce apoptosis and autophagy in renal cell carcinoma (RCC); NVP-BEZ235 combined with an autophagy inhibitor can increase the apoptosis of RCC. Lin JF et al. found that in human prostate cancer cells, benzyl isothiocyanate (BITC) can inhibit autophagy induced by the mTOR pathway, while autophagy inhibition by 3-MA can increase BITC-induced apoptosis.

The cytotoxicity of chemotherapeutic drugs in tumour cells can release high-mobility group protein 1 (HMGB1) from cells, activate autophagy by affecting the PI3Kc3/MEK/ERK pathway and mediate chemotherapy resistance; the use of RNA interference or PI3Kc3/MEK/ERK inhibitors (3-MA or U0126) can reverse the chemotherapy resistance of leukaemia. Vemurafenib is highly susceptible to drug resistance in the treatment of melanoma. Martin S. et al. found that this drug significantly activates the MAPK/MEK pathway and promotes autophagy. MEK inhibitors combined with autophagy inhibitors can further activate tumour cells. Death-related

pathways enhance the effects of chemotherapy. In summary, targeted inhibition of autophagy via the PI3K/AKT or MEK/ERK pathways can overcome chemotherapy resistance, thereby increasing the sensitivity of tumour cells to chemotherapeutic drugs.

24.2.2.2 TLR9/NF-κB Signalling Pathway

TLR9 is a member of the toll-like receptor family, and its expression level is higher in hepatocellular carcinoma, oesophageal cancer, lung cancer, breast cancer, gastric cancer and prostate cancer cells than in paracancerous cells; high expression is associated with poor prognosis. TLR9-mediated activation of the NF-κB signalling pathway and associated expression of matrix metalloproteinase-2 (MMP-2), MMP-7 and cyclooxygenase 2 mRNA are closely related to tumour progression and migration. The autophagy inhibitor CQ may inhibit this pathway by modifying the nucleic acid structure responsible for TLR activation to prevent it from binding to the TLR. CQ inhibits the invasion and viability of triple-negative breast cancer cells in vitro, but it does not prevent the growth of triple-negative breast cancer cells with high or low expression levels of TLR9 in vivo (Tuomela et al. 2013).

24.2.2.3 AMPK Pathway

AMPK, an AMP-dependent protein kinase, is a key molecule in the regulation of bioenergy metabolism. AMPK eventually converges to the mTOR pathway, thereby affecting the level of autophagy in the cell. It is known that the degree of neuroendocrine differentiation (NED) in prostate cancer is associated with chemotherapy resistance. Chang P. C. et al. found that interleukin 6 (IL-6) can induce autophagy in androgen-dependent prostate cancer LNCaP cells and exerts stronger autophagy in androgen-independent cells. Inhibition of autophagy using CQ or silencing beclin-1 or Atg5 enhances IL-6-induced apoptosis of prostate cancer cells. Further studies have found that IL-6 regulates autophagy mainly by activating the AMPK/mTOR pathway, suggesting that autophagy plays an important role in the chemotherapy resistance of prostate cancer by regulating the AMPK/mTOR pathway. Other studies have confirmed that in colon cancer cells, inhibition of eukaryotic elongation factor 2 kinase (EEF2K), a negative regulator, induces autophagy by activating the AMPK/ULK1 pathway and promotes chemotherapy resistance of tumour cells. Adriamycin resistance in breast cancer is associated with TRPC5-induced autophagy, whereas TRPC5-mediated cytoprotective autophagy is dependent on the CaMKKβ/AMPKα/mTOR pathway (Zhang and McCarty 2017).

24.2.2.4 Epidermal Growth Factor Receptor (EGFR) Pathway

EGFR is an important molecule that affects tumour cell proliferation and belongs to the tyrosine kinase receptor family. EGFR binds to its ligand to activate the phosphorylation of downstream molecules, including MAPK, Akt and JNK, which are closely related to tumorigenesis. Therefore, the EGFR pathway has become a candidate for targeted therapy. Gefitinib and erlotinib are epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) that are resistant to non-small-cell lung cancer. Han W. and X. Su et al. found that gefitinib and erlotinib induced autophagy and inhibited the PI3K/Akt/mTOR signalling pathway; inhibiting autophagy or silencing autophagy gene expression could enhance the antitumour effect of EGFR-TKIs. In addition, it is generally believed that a tyrosine kinase inhibitor alone has a limited effect on tumour killing, and studies suggest that regulation of the autophagy lysosomal pathway can effectively promote apoptosis. Kohli L. et al. attempted to provide a more effective drug combination for the treatment of patients with malignant peripheral schwannoma. It was found that the tyrosine kinase inhibitor (TKI) PD168393 induces autophagy and inhibits the activation of Akt and mTOR. CQ combined with the TKI inhibitor can enhance its toxic effects on tumour cells and lead to lysosomal dysfunction, promoting apoptosis. These results indicated that inhibition of autophagy may enhance the action of chemotherapy drugs by inhibiting the PI3K/Akt/mTOR pathway and reduce chemotherapy resistance.

24.2.2.5 JNK, p53 Pathway

Sui X. et al. found that in p53-deficient HCT116 and HT29 colon cancer cells, JNK-activated autophagy exerts a protective effect on cells while enhancing their chemosensitivity to 5-FU and induced phosphorylation of Bcl-2; an inhibitor of JNK, SP600125 or siRNA promoted apoptosis of colon cancer cells by inhibiting autophagy. This study provides a new therapeutic strategy for the improvement of 5-FU-based chemotherapy in patients with intestinal cancer with p53 mutation. As a recognized tumour suppressor gene, p53 is also involved in the regulation of autophagy. Amaravadi R. K. and other studies have found that in c-myc-activated lymphoma mouse model, inhibition of autophagy with CQ or Atg5 shRNA activates p53 and induces tumour cell death.

24.2.2.6 MicroRNAs

miRNAs are a class of small, non-coding, single-stranded RNAs that regulate the expression of genes after transcription and play an important role in regulating cell growth. A series of miRNAs have recently been reported to be involved in chemotherapy resistance. They can selectively downregulate autophagy-related genes, thereby inhibiting autophagy. Chang Z. et al. blocked autophagy by using miR-101 and enhanced the sensitivity of human osteosarcoma U-2OS cells to chemotherapy.

Downregulation of miR-30d enhances cisplatin resistance by activating autophagy in thyroid cancer ATC cells. The use of miR-30d can be a potential intervention for chemotherapy. In the hepatoma HepG2 cell line, miR-101 enhances cisplatin-induced apoptosis by inhibiting the expression of the autophagy genes RAB5A, STMN1 and ATG4D. This study suggests the effects of miR-101 and autophagy in the chemoresistance of liver cancer. In addition, early studies have found that combinated treatment with imatinib in CML, targeting miR30A inhibits autophagy, downregulates the expression of BECN1 and Atg5 and enhances chemotherapy resistance of tumour cells. In contrast, an antagonist of miR30A can enhance autophagy and inhibit apoptosis. The effects of miRNAs and autophagy in antitumour therapy need to be further elucidated, but most scholars believe that miRNA is still an important field of chemotherapy resistance, and inhibition of autophagy may help to improve the chemotherapy effect of some drugs. *Fusobacterium nucleatum* is a Gram-negative bacterium commonly found in the human oral cavity that can promote the tolerance of colorectal cancer to chemotherapy by regulating the autophagy of cancer cells. *Fusobacterium nucleatum*-mediated chemoresistance in colorectal cancer is caused by a selective reduction of miR-4802 and miR-18a* via activation of the TLR4 and MYD88 innate immune system signalling pathways, followed by activation of autophagy (Yu et al. 2017). Adult astrocytes can promote the invasiveness of brain-circulating breast cancer cells by modulating the CXCL12-miR-345-KISS1 axis to upregulate autophagy signalling pathways (Kaverina et al. 2017).

24.2.2.7 CXCL12/CXCR4 Signalling Pathway

Chemokine CXCL12 and the ligand CXCR4 are associated with cancer progression, affecting the invasive phenotype of pancreatic cancer. Studies have found that CQ and HCQ can inhibit the proliferation of pancreatic cancer cells in vitro. CQ partially inhibits the CXCL12/CXCR4 signalling pathway through extracellular signal-regulated kinase (ERK) transduction signalling and decreased phosphorylation of signal transducer and activator of transcription 3 (STAT3). CQ and HCQ can induce CXCR4 internalization in cancer stem cells, making these cells insensitive to CXCL12 signalling. Studies using xenograft models of pancreatic cancer patients have shown that CQ specifically targets highly invasive cancer stem cells by inhibiting their self-renewal process. Thus, CQ can be used to block cancer stem cell metastasis and can be combined with other anticancer agents (e.g. gemcitabine) that target most tumours.

24.2.2.8 MAPK14/p38α Pathway

Recent studies have found that the MAPK/p38 pathway is involved in the chemoresistance of tumour cells. Paillas et al. found that there is a close relationship between MAPK14/p38, autophagy and irinotecan resistance: the MAPK/p38 pathway is activated, and its induced autophagy plays a role in protecting tumour cells against

irinotecan. In addition, activation of p38 MAPK is thought to be a critical step in cellular responses to 5-fluorouracil by regulating the balance between apoptosis and autophagy.

24.2.2.9 Other Mechanisms

Wnt5A is an intrinsic factor that inhibits the expression of β-catenin, a key molecule in the canonical Wnt signalling pathway, and promotes tumour invasion. The resistance of melanoma cells to targeted therapy is related to Wnt5A expression. Melanoma cells expressing high levels of Wnt5A and low levels of β-catenin have higher levels of basal autophagy, and their sensitivity to the autophagy inhibitor Lys05 is relatively weak, while β-catenin activity can reverse this situation. Therefore, the expression level of Wnt5A should be considered when predicting and evaluating the effects of autophagy inhibitors on melanoma patients. Epithelial and endothelial tyrosine kinases (Etk) are critical for the regulation of chemoresistance in small-cell lung cancer (SCLC). Etk interacts with PFKFB4 to promote chemoresistance in SCLC by regulating autophagy. Abnormal Etk and PFKFB4 can be used as predictors of chemoresistance and potential targets for the treatment of SCLC (Wang et al. 2018).

24.2.3 Autophagic Cell Death May Enhance Chemosensitivity

The function of chemotherapy drugs is mainly to induce the apoptosis of tumour cells. However, studies on autophagy in recent years have found that chemotherapy drugs can not only induce apoptosis of tumour cells but also induce autophagic death of cells. Autophagic cell death is the result of excessive autophagy in cells. The main feature is the presence of abundant autophagic lysosomes, the degradation of most substances in the cytoplasm and the maintenance of nuclear integrity. Autophagic cell death is usually independent of the activity of the caspase family. Since autophagy and apoptosis share a common molecular pathway, autophagy and apoptosis are closely related and uncontrolled autophagy usually induces autophagic cell death in cancer cells. When tumour cells are exposed to various adverse microenvironments such as hypoxia, low nutrition, chemotherapy and radiation, autophagy can not only inhibit apoptosis and protect the survival of tumour cells but also participate in the process of inducing cell death together with apoptosis. In addition, autophagic cell death can serve as an alternative mechanism for the apoptotic deletion and resistance of tumour cells. Autophagic cell death, like apoptosis, is a proactive extinction process of the organism that is initiated to maintain homeostasis.

The antitumour effects of many chemotherapeutic drugs are often associated with autophagy and autophagy-dependent signalling pathways. Many chemotherapeutic drugs have been shown to directly or indirectly exert antitumour effects by inducing

autophagic cell death in tumours without obvious chemotherapy resistance, such as cyclosporine A, arsenic trioxide, resveratrol, actinomycete D and tamoxifen. A small-molecule compound, STF-62247, can promote the death of renal cell carcinoma cells in vitro and in vivo by inducing autophagy during the treatment of renal carcinoma. The sensitivity of STF-2247 to renal cancer cells can be reduced by inhibiting the autophagy gene Atg7 or Atg9 with small interfering RNA. Studies have shown that low doses of cyclosporine A can increase the autophagic cell death induced by arsenic trioxide. Clofarabine has a significant inhibitory effect on the proliferation of AML cells, and its mechanism may be to induce the autophagic death of U937 cells by upregulating the expression of Beclin 1 protein. RAD001, an mTOR inhibitor, can promote autophagic cell death induced by temozolomide in glioma. Fk-16, which is derived from the antitumour peptide ll-37, can induce caspase-independent apoptosis and autophagic cell death in colon cancer. Sc-59, which is a novel sorafenib derivative, and sorafenib induce dose- and time-dependent autophagic cell death in hepatocellular carcinoma cells. Tamoxifen (TAM) can induce apoptosis and autophagic cell death in human breast cancer cells by increasing the expression of beclin-1. TAM-induced cell death was significantly reduced when autophagy was blocked by 3-MA. Resveratrol, a natural polyphenol, activates autophagic cell death in prostate cancer cells by downregulating the STIM1 and mTOR pathways. Multidrug resistance (MDR) occurs in most patients with colon cancer. Therefore, the reversal of MDR plays an important role in the success of chemotherapy for colon cancer, and now the most common method is to inhibit the apoptosis of cancer cells. Tanshinone can inhibit the proliferation of anti-apoptotic colon cancer cells by inducing autophagic cell death and p53-independent cytotoxicity. Isogambogenic acid exerts an anticancer effect on human NSCLC, mainly by inducing apoptotic-independent autophagic cell death in NSCLC cells. Psammaplin A, a natural product isolated from sponge, has an anticancer effect by inducing cell cycle arrest and apoptosis. Psammaplin A can also overcome multidrug resistance in doxorubicin-resistant breast cancer cells by Sirtuin-1-mediated autophagic cell death. Luminacin, a natural product extract from Streptomyces species marine microbes, can inhibit the growth and progression of head and neck squamous cell carcinoma by inducing autophagic cell death. Dihydroartemisinin (DHA) exerts an antitumour effect by inducing apoptosis and autophagic cell death in the mitochondria and endoplasmic reticulum of human glioblastoma cells. YM155, a kind of selective survival protein inhibitor, has an anticancer effect on head and neck squamous carcinoma by facilitating the dual induction of apoptosis and autophagic cell death. Flavokawain B (FKB), a natural extract of the piperaceae plant kava, can induce autophagic cell death and inhibits xenograft tumour growth of human gastric cancer cells in nude mice by a ROS-mediated signalling pathway.

Autophagic cell death is also an alternative pathway for the death of tumour cells with apoptotic deletion. Studies have found that when apoptosis is blocked by the caspase inhibitor z-VAD, and autophagy is inhibited by the autophagy inhibitor 3-MA and wortmannin or RNAi knockout of the Atg7 or beclin-1 gene, non-apoptotic cell death is reduced. The results indicate that inhibition of caspase blocks apoptosis but also simultaneously activates autophagic death. When the Atg5 or beclin1 gene was

knocked out in $Bax^{-/-}Bak^{-/-}$ mouse embryonic fibroblasts (MEFs) treated with the apoptosis-inducing agent etoposide or staurosporine, non-apoptotic death of the cells was observed, accompanied by a decrease in autophagy vacuoles. When apoptosis is blocked, the inhibition of apoptosis leads to an increase in autophagic cell death. The results suggest that autophagy can promote cell death in the absence of apoptosis. 5-FU could reduce the proliferation rate of $PUMA^{-}$ or Bax^{-} human colon cancer cells. The autophagic signalling pathway was activated in apoptotic-deficient colon cancer cells, and the mortality of cancer cells was reversed after inhibition of autophagy. The study showed that apoptotic-deficient colon cancer cells enhanced chemosensitivity to 5-FU through autophagic cell death.

FOXO3a, a transcription factor, recently was found to connect autophagy to apoptosis. FOXO3a is itself turned over by basal autophagy, creating a potential feedback loop. Increased FOXO3a upon autophagy inhibition stimulates transcription of the pro-apoptotic BBC3/PUMA gene to cause apoptosis sensitization. Autophagy inhibition can sensitize tumour cells to chemotherapy drugs and allows an autophagy inhibitor to change the action of an MDM2-targeted drug from growth inhibition to apoptosis, reducing tumour burden in vivo. This study showed that turning off autophagy can promote the sensitivity of some cancer cells to chemotherapy drugs or radiation (Fitzwalter et al. 2018). The combination of mTOR and lysosome inhibitor treatment of a human renal cell line resulted in RIPK1- and oxidative stress-dependent necrotic apoptosis. The results indicated that autophagy can inhibit necrotic apoptosis through the degradation of RIPK1 and the reduction of reactive oxygen species (ROS). Inhibiting the autophagy of dormant breast cancer cells can significantly inhibit the survival time of cancer cells and the formation of metastases in a preclinical 3D model of breast cancer dormancy. In vivo experiments have found that Atg7 is necessary to activate autophagy. Mitochondrial damage and the accumulation of ROS were initiated by the inhibition of the autophagic pathway and induced the apoptosis of dormant breast cancer cells.

Autophagy has a similar killing effect on chemotherapy-resistant tumour cells. SAHA, an HDAC inhibitor, can induce autophagic cell death of tamoxifen-resistant human breast cancer MCF-7 cells. There are a large number of autophagosomes in cancer cells according to electron microscopy results, and this autophagic death can inhibit tumour growth both in vitro and in vivo. NVP-BEZ235, a PI3K inhibitor, can inhibit cisplatin-resistant urothelial tumour cell proliferation and enhances its sensitivity to cisplatin by inducing autophagy and arresting the cell cycle. The results suggest that autophagy can also promote death in certain chemotherapy-resistant tumour cells. ARHI (DIRAS3), an imprinted tumour suppressor gene, is downregulated in 60% of ovarian cancers. The chemosensitivity of ovarian cancer to cisplatin in cell lines and xenografts models was enhanced by autophagy-related cell death mediated by ARHI (DIRAS3).

Multiple signalling pathways have been demonstrated to be involved in the regulation of autophagic cell death, including JNK-cJun, p38, Notch and AMPK/AKT1/mTOR. Li et al. found regulation between JNK-cJun and beclin-1 at the transcriptional level. Ceramide can upregulate the expression of beclin-1 at the mRNA and protein levels. Ceramide also can promote tumour cells to activate

JNK, enhance the phosphorylation of c-Jun, regulate the transcription of beclin-1 and, ultimately, induce autophagic cell death. Wang et al. found that curcumin and berberine exerted synergistic chemoprophylactic effects on human breast cancer cells by inducing apoptosis and autophagic cell death, and the JNK/Bcl-2/beclin1 pathway played a key role in inducing the autophagic cell death of breast cancer cells. Simone C. et al. found that the p38 inhibitor SB202190 or gene knockout of p38α can arrest the cell cycle and induce autophagic cell death in colon cancer cell lines. Rhus Coriaria, an ethanol extract from the lacquer tree with strong anticancer activity in breast cancer, can induce the senescence and autophagic cell death of breast cancer cells by activating the p38 and ERK1/2 signalling pathways. Polyphyllin VII, a saponin extracted from a traditional Chinese herb, can induce autophagic death in human hepatocellular carcinoma HepG2 cells by activating the JNK pathway and inhibiting the PI3K/AKT/mTOR pathway. Cardamonin, a natural extract from a traditional Chinese herb, can induce autophagy and produce anticancer effects in human colorectal cancer HCT116 cells by activating the JNK pathway. 6-Shogaol, a gingerol extract from ginger, can inhibit the proliferation of breast cancer cells and stem cell-like globules by regulating the Notch signalling pathway and inducing autophagic cell death. Dehydroepiandrosterone (DHEA), an endogenous hormone with anticancer activity, can induce autophagic cell death in human hepatocellular carcinoma HepG2 cells by promoting the expression of p62 through activation of the JNK–Nrf2–p62 signalling pathway. CYT-Rx20, a synthetic β-nitrostyrene derivative, can induce breast cancer cell death and autophagy through the MEK/ERK pathway in a ROS-mediated manner. Raloxifene, a selective oestrogen receptor regulator, can induce autophagy by sensing ATP reduction and activation of AMPK. Over-activated autophagy of breast cancer cells promotes cell death and mediates the anticancer effect of raloxifen.

The AMPK/AKT1/mTOR pathway is also an important pathway regulating autophagic cell death. Tanshinone IIA can induce autophagic cell death in KBM-5 leukemic cells by activating AMPK/ERK and inhibiting mTOR and p70 S6K activity. Baicalein has good antitumour activity and can induce autophagic death in human tumour cells by activating the AMPK/ULK1 pathway and inhibiting the expression of the mTOR/Raptor complex. Gefitinib can induce autophagic cell death by upregulating AMPK in human lung cancer cells, and its receptor antagonist or inhibition of AMPK with siRNA can block the autophagic cell death induced by gefitinib. Chlorpromazine, an antipsychotic drug, can induce autophagic death in human glioma U-87MG cells by inhibiting the Akt/mTOR pathway. Tamoxifen and fluvastatin are mainly used in endocrine therapy for ER^+ breast cancer patients but are prone to endocrine drug resistance. Silencing microRNA-21 increases autophagic cell death in breast cancer cells by inhibiting the PI3K/AKT/mTOR pathway, thus enhancing the sensitivity of ER^+ breast cancer cells to tamoxifen and fulvestrant. Honokiol, a natural extract from the bark of *Magnolia officinalis* with an anticancer effect, can induce autophagic cell death in malignant glioma through the p53/PI3K/Akt/mTOR signalling pathway mediated by ROS. G9a is highly expressed in bladder transitional cell carcinoma, and its inhibition significantly reduced the proliferation of bladder cancer cells by inducing autophagic cell death through the AMPK/mTOR signalling pathway. Oleanolic acid exerts anticancer effects by inducing autophagic

cell death in hepatocellular carcinoma cells through the PI3K/Akt/mTOR and ROS-dependent pathways. Hernandezine is an alkaloid isolated from a Chinese herb. As a new AMPK activator, hernandezine can induce autophagic cell death in many drug-resistant cancer cell lines by directly activating AMPK. Carnosic acid, a polyphenol isolated from rosemary, can induce autophagic cell death by inhibiting the Akt/mTOR pathway in human hepatocellular carcinoma cells and has the potential to treat hepatocellular carcinoma by inducing autophagy. Andrographolide analogues can induce apoptosis and autophagic cell death in human leukaemia U937 cells by inhibiting the PI3K/Akt/mTOR pathway. SCD1, a key regulator in the progression of cancer, can induce autophagic cell death in human hepatocellular carcinoma by negative regulation through inactivation of the AMPK signalling pathway. CAB, a first-line drug for the treatment of prolactinoma, can mediate the shrinkage of prolactinoma by inhibiting the mTOR signalling pathway and inducing autophagy-dependent cell death.

Becn1 (beclin1), the only confirmed "autophagy gene" in mammals, is the direct executor of autophagy and the essential molecule for the formation of autophagosomes. The downregulation of microRNA-506 expression is associated with the progression of human pancreatic ductal adenocarcinoma, which induces the autophagic cell death of pancreatic cancer cells by directly targeting the STAT3–BCL2–BECN1 pathway (Sun et al. 2017). Genetic targeting of the autophagy gene Becn1 in B16-F10 tumours inhibits their growth by inducing a massive infiltration of functional natural killer (NK) cells into the tumour bed. Such infiltration is primarily due to the ability of Becn1-defective tumour cells to overexpress and release CCL5 cytokine in the tumour microenvironment by a mechanism involving the activation of the MAPK8/JNK-JUN/c-Jun signalling pathway. A significant increase in survival is found in melanoma patients expressing a high level of CCL5 (Noman et al. 2018). Λ-WH0402, a novel ruthenium complex, can induce death in human hepatocellular carcinoma LM6 cells by triggering the Becn1-dependent autophagy pathway.

Autophagy exerts protective or killing double effects in tumours and influences the occurrence and progression of tumours by promoting or inhibiting effects. Whether tumours escape from a harsh microenvironment by autophagy or the initiation of autophagic cell death may be related to various factors such as the characteristics of tumour cells and the specific autophagy process. All of these questions need to be studied further.

24.3 The Function of Autophagy in Tumour Stem Cell-Mediated Chemoresistance

To solve the problem of chemotherapy resistance, recurrence and metastasis in cancer patients, it is particularly important to understand the origin of tumours. Recently, the clonal evolution model and the cancer stem cell model have been

regarded as two mainstream theories in academia. The latter explains that malignant tumours come from a small number of cancer stem-like cells, cancer-initiating cells (CIC) or cancer stem cells (CSCs). Since the first discovery of cancer stem cells in human subacute myeloid leukaemia, CSCs have been recognized as a major cause of anticancer therapy failure. Because traditional anticancer therapies are designed to kill normal cancer cells rather than CSCs, residual CSCs can lead to poor prognosis such as chemotherapy resistance, tumour recurrence and metastasis. CD44(+)/CD24(−)/epithelial-specific antigen(+) cells have the ability to self-renew, and they can preferentially survive chemotherapy, producing chemotherapy resistance. This phenomenon has been confirmed in many studies. Levina et al. found that treatment of residual tumour cells with different chemotherapeutic drugs such as doxorubicin, cisplatin and etoposide induces obvious stem cell characteristics. These cells express stem cell markers such as CD133, CD117, CD117, SSEA-3, OCT-4 and β-catenin on their surface, with deleted expression of differentiation markers such as cytokeratin 8/18. Lung cancer cells that are resistant to chemotherapy have the ability to self-renew and differentiate, and these cells were inoculated into SCID mice to confirm their high tumorigenicity and metastasis. This study supports the speculation that chemotherapy-resistant tumour cells are derived from cancer stem cells. Dylla et al. enriched the colon cancer stem cell (CoCSC) subpopulation, which has an ESA (+) CD44 (+) phenotype after chemotherapy and shows strong tumorigenicity and tumorigenic ability. It was found that CoCSC, which is enriched after colon cancer chemotherapy, has a tumour phenotype similar to that of the parent. Further, the expression of ALDH1 was found to be elevated by detecting the biological characteristics of CoCSC. ALDH1 is an important molecule that mediates chemotactic resistance to cyclophosphamide. The results suggest that non-tumorigenic tumour cells are more sensitive to chemotherapeutic drugs, while tumour cells with a stem cell phenotype are prone to chemotherapy tolerance. In addition, Vincent Z. et al. found that CD133 (+) stem cells derived from the colon cancer cell line Colo205 have specific proteomic characteristics that are closely related to the occurrence of cisplatin resistance. Yu D. et al. found that in the human laryngeal cancer Hep-2 cell line, CD133 (+) stem cells were more tolerant of 5-FU chemotherapy under chemotherapy drug treatment. The bmi-1 gene, which plays an important role in stem cell self-renewal, is also highly expressed in CD133+ cells. Deepening the understanding of cancer stem cells, studying how cancer stem cells escape the attack of chemotherapy drugs and effectively combining the strategies of targeting interventional stem cells and traditional anticancer therapy are new trends for future anticancer treatment.

Cancer stem cell involvement in chemotherapy resistance is a major problem in cancer treatment and is also an important factor affecting the prognosis of patients. Current research on cancer stem cells has been very active, but no highly effective method for interfering with CSCs has been found. The review by Pindiprolu et al. mentioned that the presence of breast cancer stem cells (BCSCs) is a major cause of tumour recurrence, metastasis and chemoresistance in breast cancer. Now, molecular targets for some breast cancer stem cells that are expected to become pharmacological targets have been identified, including surface markers, self-renewal pathways,

apoptotic pathways, autophagy, metabolism and microenvironment (Pindiprolu et al. 2018). Li et al. showed that targeting glutaminase 1 by increasing ROS and inhibiting the Wnt/β-catenin pathway can attenuate the stemness of hepatocellular carcinoma (Li et al. 2019). La Noce et al. also found for the first time that HDAC in osteosarcoma can inhibit the expansion of CSCs and is a key factor regulating CSC phenotype and cancer growth in vivo. Thus, HDAC2 can be identified as a potential therapeutic target in the treatment of human osteosarcoma (La Noce et al. 2018). Studies by Bishnu et al. have also found that metformin inhibits the development of cancer stem cells by regulating the differentiation of cancer stem cells in ovarian cancer cells (Bishnu et al. 2019).

Studying the mechanisms by which cancer stem cells escape chemotherapy drugs and improving chemotherapy sensitivity are critical for the effective treatment of cancer, but the mechanism of drug resistance is not clearly understood. Numerous studies of various cancer types and responses to cancer therapies have confirmed that autophagy is both induced by the treatment drug and resistant to treatment (Galluzzi et al. 2017; Levy et al. 2017), highlighting the importance of exploring tumour resistance mechanisms that already result in high autophagy levels. Recently, Yu et al. published an article to demonstrate the mechanism of chemotherapy resistance. According to this article, the intestinal microbial *Fusobacterium nucleatum* can cause chemoresistance by regulating autophagy (Yu et al. 2017). This major finding suggests that autophagy may be an important mechanism for cancer stem cells to escape chemotherapy drugs. Peng et al. suggest that autophagy, through the participation of FOXA2, maintains the characteristics of ovarian cancer stem cells. Therefore, autophagy and FOXA2 are potential targets for ovarian cancer stem cell directed therapies (Peng et al. 2017). Autophagy levels are also selectively elevated in cisplatin-resistant bladder cancer cells, and autophagy inhibition can specifically decrease drug-resistant bladder cancer. Elevated MST4 activity induces phosphorylation and activation of ATG4B protease, resulting in increased autophagy flux, increased self-renewal properties and globular formation in glioma stem-like cells (GSC), and increased tumorigenicity in vivo. Direct targeting of ATG4B or inhibition of autophagy with the autophagy inhibitor chloroquine promoted the therapeutic effect of radiation in a glioblastoma (GBM) transplantation model. This effect is related to the loss of GSC self-renewal ability. Consistent with these findings, the levels of MST4, phospho-ATG4B and LC3B staining were inversely correlated with GBM patient outcomes (Huang et al. 2017). These findings suggest that there are specific molecular mechanisms that promote autophagy induction in tumour cells, but at least some of these mechanisms are CSC-specific and may help explain how CSC becomes a key mediator of drug resistance.

In summary, this growing body of research shows that autophagy has a cytoprotective effect, which provides a powerful theoretical basis for combining cancer treatment with drugs that inhibit autophagy (Levy et al. 2017; Drake et al. 2017).

24.4 Tumour Microenvironment-Induced Autophagy and Chemoresistance

The interaction between cells and their microenvironment plays a critical role in cellular growth and the maintenance of homeostasis. In the past, research and treatment of tumours often focused on the tumour cells themselves, while ignoring the effects of the microenvironment. According to a widely accepted concept, the soil (microenvironment) and seed (cancer cell) hypothesis, tumour cell–microenvironment interactions not only play a pivotal role in tumour initiation, development and metastasis but also influence the cancer response to anticancer therapies. The tumour microenvironment consists of tumour cells, surrounding blood vessels, other non-cancer cells and soluble factors.

Hypoxia is a common feature of malignant tumours. The hypoxic microenvironment of a tumour is a state of low oxygen resulting from the imbalance in oxygen need and supply due to the high consumption of oxygen by rapidly proliferating cells, stimulating angiogenesis and hypervascularization to bring oxygenated blood to the tumour. However, the new vasculature is often structurally and functionally abnormal. Therefore, this causes diminished blood flow and an inadequate supply of nutrients and oxygen for tumour cells. In addition, the disease itself or treatment-related anaemia can result in the ischaemic and hypoxic state of the tumour tissue. The ischaemic and hypoxic microenvironment is not only closely related to the prognosis of patients but also leads to drug resistance in tumour cells.

Hypoxia affects the efficacy of cancer cell chemotherapy, and the survival rate of patients with severely hypoxic tumours is shorter than that of patients with less hypoxic tumours. To study the impact of hypoxia on cancer cells, researchers mimicked the hypoxic tumour microenvironment and found that cancer cells showed chemoresistance. It has been reported that hypoxia induces the resistance of a number of tumour cell types to cisplatin, doxorubicin, 5-flouoruracil and gemcitabine.

Hypoxia-inducible factor-1 (HIF-1) is an important factor in the adaptation of cells to the hypoxic microenvironment. Hypoxia, through HIF-1, is known to contribute to the chemoresistance of a number of different tumours. Additionally, HIF-α regulates the expression of many genes and proteins in cancer cells. A paper reported that HIF-1α plays an important role in the regulation of autophagy-related genes. Mazure et al. found that BNIP3 and BNIP3L, two HIF-targeted genes, can compete with the Beclin 1-Bcl-2 and Beclin 1-Bcl-X(L) complexes, releasing Beclin 1 and then enhancing autophagy. Studies in cervical cancer HeLa cells have found that inhibition of HIF-1α can reduce autophagy levels in cells. Yang et al. showed that hypoxia-induced autophagy enhanced the gemcitabine resistance of bladder cancer cells through HIF activation. Huang et al. found that hypoxia-induced autophagy via the HIF-1α/miR-224-3p/Atg5 axis affects cell chemosensitivity in glioblastoma and astrocytoma (Huang et al. 2018). Liu et al. found that hypoxia caused human cervical cancer HeLa cells to be resistant to chemotherapy with N-4-hydroxyphenyl retinoid (4-HPR). The main mechanism is the upregulation of autophagy, and further inhibition of autophagy with 3-MA or CQ significantly increases apoptosis. This study

suggests that autophagy mediates hypoxia-induced cervical cancer cell resistance to 4-HPR chemotherapy. Our lab also confirmed that the hypoxic microenvironment of liver cancer can induce autophagy and promote the chemotherapy resistance of liver cancer cells. Compared with normoxic levels, the apoptosis of tumour cells induced by chemotherapy was significantly reduced in the hypoxic microenvironment. In contrast, after autophagy was inhibited by 3-MA or beclin1 siRNA, cell apoptosis was reversed, indicating that inhibition of autophagy increased the chemosensitivity of hepatoma cells. In addition, the hypoxic microenvironment of tumours is often accompanied by a lack of nutrition. Our lab found that autophagy is not only involved in the chemoresistance of hepatocellular carcinoma cells in a hypoxic microenvironment but also plays a protective role in the induction of apoptosis induced by nutrient deficiency, which relies on the activation of the autophagy gene beclin-1.

In contrast to the above results, however, it has also been reported that hypoxia-induced autophagy is not involved in the activation of HIF-1 and the expression of the HIF-1 target genes BNIP3 or BNIP3L but is induced by the AMPK pathway. Other studies suggest that HIF-1α-mediated autophagy activated by glycolysis promotes the chemotherapy resistance of paclitaxel to cervical cancer. In addition, activating transcriptional factor 4 (ATF4), which affects drug resistance, regulates chemoresistance with autophagy. ATF4 is upregulated under the action of a hypoxic tumour microenvironment and endoplasmic reticulum stress. The increased expression of ATF4 is also associated with resistance to many chemotherapeutic drugs, including DNA-damaging drugs, non-steroidal anti-inflammatory drugs and protease inhibitors. Rzymski T et al. found that ATF4-induced autophagy protects against the apoptosis of tumour cells induced by proteasome inhibitor bortezomib (PS-341) under hypoxic conditions and alleviates the overload of bortezomib on cells, thereby inducing chemotherapy resistance.

Mesenchymal stem cells (MSCs) also play an important role in the tumour microenvironment. Piya reported that the co-culture of AML cells with mesenchymal stromal cells (MSC) induces autophagy in AML cells and promotes chemoresistance of cells to cytarabine and Idarubicin; furthermore, the inhibition of autophagy in AML enhances sensitivity to drugs, and sensitivity was more pronounced with concomitant inhibition of autophagy in AML and MSCs. Thus, MSCs mediate the chemoresistance of AML cells by autophagy (Piya et al. 2017). Yang found that MSCs mediate the chemotherapy resistance of multiple myeloma (MM). Mechanistically, MSCs promote the expression of autophagy-related genes to activate autophagy. Autophagy inhibition prevented MSC-induced chemoresistance (Yang et al. 2017).

It is well known that chronic inflammation is involved in tumour progression. Additionally, evidence shows that inflammation can activate autophagy. IL-6, an inflammatory autocrine and paracrine cytokine, is overexpressed in the tumour microenvironment. Xue et al. indicated that IL-6 is a potent initiator of autophagy in glioblastoma, and blockade of IL-6 inhibits autophagy, promoting tumour apoptosis. Zhang et al. showed that IL-6-mediated autophagy was involved in the chemotherapy resistance of Mantle cell lymphoma cells. Most strikingly, autophagy also can augment IL-6 secretion (Zhang and McCarty 2017). Thus, interruption of the

IL-6- autophagy network could reduce tumour drug resistance. The interleukin-17/interleukin-17 receptor (IL-17/IL-17 R) complex has been shown to be an important regulator of inflammation. It was found that IL-17/IL-17 R induced autophagy, which was shown to promote resistance to oxaliplatin in HCC.

In addition, studies by Zhao X. and others have found that the tumour stroma in the microenvironment can induce autophagy. This new model is called 'tumour interstitial autophagy'. The tumour stroma is a complex, three-dimensional chamber composed of tumour-associated fibroblasts, extracellular matrix, immune cells, endothelial cells and the like. The stroma affects tumour growth, proliferation and invasion around parenchymal cells. Due to the increased tumour volume and lack of nutrient supply, the tumour stroma is also in a hypoxic, high metabolic stress and low-pH environment. These stress conditions are sufficient to activate the occurrence of autophagy. Tumour interstitial autophagy can affect tumours, including tumour cell survival, microenvironment remodelling, anti-apoptosis and DNA damage, genetic instability and the stem characteristics of tumour cells.

At present, few studies have examined the effect of tumour interstitial autophagy on chemotherapeutic drug resistance. The discovery of tumour interstitial autophagy is mainly based on the identification of the interstitial marker caveolin-1 (Cav-1). It has been found that the loss of Cav-1 expression in the stroma is a powerful prognostic indicator of primary breast cancer and prostate cancer. In ER(+) breast cancer, the loss of Cav-1 is closely related to lymphatic metastasis, early recurrence and resistance to tamoxifen. In addition, related studies suggest that the loss of Cav-1 in the stroma is a biomarker of chronic hypoxia, oxidative stress and autophagy in the tumour microenvironment. MCF-7 breast cancer cells mainly destroy the expression of Cav-1 by lysosomal degradation and the autophagy pathway and stimulate the oxidative stress response of adjacent fibroblasts, resulting in an abnormal tumour-associated fibroblast phenotype. Based on this, researchers developed a combination mode (tamoxifen + dasatinib) that effectively kills fibroblasts present in MCF-7 cells; this combination of drugs induces the Warburg effect in MCF-7 cells, causing tumour cell apoptosis. In the future, this 'synthetic lethal' programme may be a new treatment for clinically overcoming chemotherapy resistance.

24.5 Conclusion

With the deepening of the current body of research, we found that autophagy is inextricably linked with the occurrence of tumours, playing the role of 'double-edged sword' in the process of chemotherapy. Whether chemotherapy drugs induce cell survival or death depends mainly on the type of tumour cell, the degree of differentiation and the type of chemotherapy drug. The mechanism by which autophagy switches between tumour cell survival and death remains unclear; the correlation between autophagy and tumour stem cells, the tumour microenvironment and the detailed mechanisms remains to be elucidated. Studying the relationship between

tumour chemotherapy and autophagy, the relationship between autophagy and apoptosis and the regulation of autophagic death-related gene expression has important clinical significance for antitumour therapy. In addition, existing research has shown good application prospects in some cases. Under certain conditions, we can increase the chemosensitivity of tumour cells by using autophagy to modulate the drug and change the level of autophagy in tumour cells, thereby improving the chemotherapy effect. In short, although there is still a certain distance to traverse before the actual clinical use of autophagy-regulating drugs is achieved, we expect that as the research progresses, the mechanism will be gradually clarified, experiments will continue and experience will accumulate gradually. In the future, cancer patients will experience the ideal use of autophagy to modulate drug treatment.

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Chapter 25
Autophagy and Tumour Radiotherapy

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Abstract Radiotherapy is an important component of cancer treatment modalities. With the rapid development of three-dimensional conformal, intensity-modulated, image-guided radiotherapy and the efficacy of radiotherapy continues to improve. Autophagy, as a catabolic process, is characterized by the formation of a double-membrane vesicle. Radiotherapy is known to induce autophagy in both cancer and normal cells. Here, we reviewed the interaction of radiotherapy and autophagy in the process of cancer treatment. The potential role of autophagy modification in enhancing radiotherapy treatment will also be reviewed.

Keywords Radiotherapy · Autophagy · Radiotherapy efficacy · Cancer treatment

Tumour radiotherapy is a method for treating malignant tumours by using X-rays, electron beams, proton beams and other particle beams generated by radiation, such as radioisotope α, β and γ rays, and various X-ray treatment machines or accelerators. Tumour radiotherapy is an important component of the comprehensive treatment of malignant tumours, and it is the main treatment for tumours requiring surgical treatment and chemotherapy. Radiation therapy is widely used to treat malignant tumours, including head and neck cancer, breast cancer, lung cancer, oesophageal cancer, rectal cancer, cervical cancer, prostate cancer, melanoma, soft tissue sarcoma, lymphoma, etc. According to statistics, 65–75% of cancer patients have received radiotherapy during the treatment process. The application of radiotherapy has become more extensive, and its position in cancer treatment is becoming more prominent.

Autophagy is a catabolic pathway characterized by the formation of a double-membrane vesicle, called the autophagosome, which engulfs cytoplasmic components and delivers them to lysosomes for degradation. Autophagy is a conserved proteolytic mechanism that degrades cytoplasmic material, including cell organelles, and

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is important in maintaining intracellular homeostasis and keeping the cell healthy; it can be activated as an adaptive response to adverse environmental conditions, such as deprivation of nutrients, hypoxia and different types of therapeutic stress. Autophagy is closely related to tumours. Different stages of tumorigenesis and development are always accompanied by autophagy. Research on the relationship between autophagy and tumour radiotherapy is also widely carried out. However, different research institutions have made different conclusions regarding the role of autophagy in different tumours. Therefore, we will discuss the clinical significance of autophagy during tumour radiotherapy and the possible mechanism of autophagy in determining the efficacy of tumour radiotherapy.

25.1 The Form of Autophagy Induced by Radiotherapy in Tumour Cells and Its Clinical Significance

In recent years, with the emergence of three-dimensional conformal, intensity-modulated, image-guided radiotherapy and other precise radiotherapy techniques, radiation therapy can increase the dose to the tumour target area without increasing normal tissue damage, thereby improving the efficacy of radiotherapy. However, the tolerated radiation dose for normal tissue remains an insurmountable dose-limiting factor in conventional external radiation therapy. Therefore, finding high-efficiency and low-toxicity radiosensitizers to greatly improve the efficacy of radiotherapy without increasing normal tissue damage will become another focus of tumour radiotherapy.

In a certain way, autophagy, as a potential protective response of tumour cells to chemoradiotherapy, has become a popular research topic. Autophagy inhibitors may represent a new radiotherapy sensitizer intervention for enhancing antitumour drugs and radiotherapy. The efficacy of such an approach has opened a new era (Yang et al. 2011; Sui et al. 2013). Recently, various clinical trials have combined the autophagy inhibitor chloroquine or hydroxychloroquine with conventional treatment. Although these trials have yielded some good results and are considered to be important breakthroughs in cancer therapy, the evidence based on preclinical animal model trials is inadequate, and we are sceptical of the combination therapy approach (Bristol et al. 2013). Whether autophagy induced by tumour radiotherapy protects cells or increases their cytotoxicity is inconsistent. Tumour cell stress caused by radiotherapy can induce autophagy forms with different functions, and we discuss these different forms of autophagy below.

25.1.1 Cytoprotective Autophagy

Studies have shown that radiotherapy can induce protective autophagy in tumour cells, maintain the stability of the internal environment and enable tumour cells to adapt to the harsh living environment and continue to survive. Bristol et al. found that radiotherapy of MCF-7 breast cancer cells induces cytoprotective autophagy, and drug-inhibited radiotherapy-induced tumour cell autophagy can lead to cell death. Chakradeo et al. (2015) believe that p53 plays an important role in protective autophagy, and knocking out the p53 gene induces protective autophagy to become into non-protective autophagy. Therefore, the autophagy induced by radiotherapy is protective or non-protective based on the activation state of the p53 gene, and p53 gene inhibition enhances the efficacy of chemotherapy (Bristol et al. 2013). Lu et al. also believe that radiotherapy-induced autophagy can promote the survival of Eca-109 oesophageal cancer cells and can significantly inhibit the survival of oesophageal cancer cells after inhibition of autophagy (Lu and Xie 2016). Additionally, many studies are showing that radiotherapy can promote protective autophagy in tumour cells (Chen and White 2011; Murrow and Debnath 2013; Wilson et al. 2011; Bristol et al. 2012). Protective autophagy is defined as an autophagy-inhibiting drug (such as chloroquine, bafilomycin, 3-methyladenine or ammonium chloride), gene silencing or autophagy-related gene knockout (e.g. Beclin, Atg5, 7 or 12) that increases the sensitivity of tumour cells to the induction of autophagy and promotes apoptosis. However, these methods require vigilance because although inhibition of autophagy can often lead to the promotion of apoptosis, there is insufficient evidence that autophagy is cytoprotective unless it can be demonstrated that when autophagy is blocked, tumour cell sensitivity to radiation has actually increased (by cloning survival assays). That is, it may be that the response to treatment-induced growth arrest or cell death is actually mediated through autophagy, but now it is converted to an apoptosis-mediated response with no real change in therapeutic sensitivity. Based on the premise that intervention or inhibition of autophagy enhances the response to treatment, a large number of clinical trials have recognized the existence of protective autophagy. However, returning to the selectivity problem mentioned earlier, autophagy is also inhibited in normal cells if the system is given tumour cell autophagy inhibitors. Given that some neurodegenerative diseases are characterized by autophagy defects (Levine 2007), it is reasonable to assume that inhibition of autophagy will impair the function of normal tissues. Even if inhibition of autophagy alone is not harmful to the patient, radiotherapy promotes protective autophagy in tumour cells, which may be the same as that in normal cells. Therefore, it can be expected that inhibition of autophagy will be secondary to increased toxicity to sensitize normal tissues such as bone marrow.

In one study, it was found that ionizing radiation promoted autophagy in breast cancer cells, and inhibition of autophagy did not alter sensitivity to radiation. Further studies showed that chloroquine did not alter mouse mammary tumours in an animal model with normal immune function. 4T1 cells are sensitive to radiation therapy

(Ko et al. 2014). Although it is not possible to determine whether radiation promotes autophagy or chloroquine effectively inhibits autophagy in tumour-bearing animals, the mechanism may be associated with the lack of sensitivity. To some extent, Kroemer's laboratory results indicate that autophagy inhibitors interfere with the immune system recognizing the ability of a tumour to develop a stress response. It has also been found in some other reports that the strategy for inhibiting autophagy in tumour-bearing animals is ineffective. Therefore, we believe that in addition to other problems, if the cytoprotective autophagy found in preclinical studies is transformed into clinical studies aimed at developing effective autophagy inhibitors for tumour patients, the primary problem to be solved is that to determine whether conventional treatment regimens promote protective autophagy in cells, it is necessary to determine a specific treatment or combination therapy to promote cytoprotective autophagy before giving autophagy inhibitors to tumour patients.

25.1.2 Cytotoxic Autophagy

Studies have found that many tumour cells destroy the self-renewal ability of cells after ionizing radiation, and mitosis of the cells is blocked, stimulating excessive autophagy of the cells and causing autophagic death. This autophagy state is regulated by IKK and NF-κB and can inhibit tumour growth. Qi Yali et al. irradiated human breast cancer cells treated with autophagy inhibitors and autophagy inducers with the same dose of X-rays and found that ionizing radiation combined with an autophagy inducer can promote cancer cell apoptosis, while ionizing radiation combined with autophagy inhibitors is able to inhibit cancer cell apoptosis. Ionizing radiation can induce autophagy and promote the apoptosis of human breast cancer cells.

On the other hand, autophagy can be induced by combining two different drugs to increase autophagic cell death. Recent studies by Gewirtz et al. have shown that vitamin D (or vitamin D analogue, EB1089) combined with radiotherapy can promote cytotoxic autophagy in breast cancer cells, and cells are more sensitive to radiotherapy. Other laboratories have also reported the occurrence of autophagy in cytotoxic cells, which either kills the cells themselves or is a precursor to apoptosis. In this case, it is important to note that different forms of autophagy are currently distinguished primarily by their functional characteristics, rather than based on morphology, biochemistry or molecular markers. Functionally, cytotoxic cellular autophagy is associated with a decrease in the number of viable cells and/or a reduction in surviving clones after treatment. Fundamentally, the difference between cytotoxic autophagy and cytoprotective autophagy is that when cytoprotective autophagy is inhibited, cells are sensitive to treatment; conversely, when cytotoxic autophagy is inhibited, treated cells are not so sensitive. This situation seems to be intuitive. When autophagy shows cytotoxicity, this autophagy is widespread and long term. However, there is no data to support this argument. We speculate that the differences in autophagy may be related to specific signals and are associated with pathways and/or autophagy transport substrates.

25.1.3 Cytostatic Autophagy

A recent study by Gewirtz Laboratories aimed to extend the discovery of enhanced radiation sensitivity of vitamin D in breast cancer cells to autophagy found in non-small-cell lung cancer cells, another form of 'cytostatic autophagy'. This form of autophagy is completely defined by function and experience relative to other forms of autophagy that we have already discussed. Vitamin D (or a vitamin D analogue, EB1089) combined with radiotherapy resulted in more pronounced growth inhibition of non-small-cell lung cancer cells than radiotherapy alone. Radiotherapy alone inhibited proliferation and significantly altered the clone survival curve, indicating increased radiation sensitivity. Similar to the effects of cytotoxic autophagy in breast cancer tumour cells, autophagy drug inhibitors, chloroquine or 3-MA protect vitamin D combined with radiosensitized cells. This form of autophagy may be closely related to the long-term growth arrest associated with ageing, and further research is needed.

Autophagy that occurs under conditions of nutrient deficiency allows cell survival to remain in a stagnant state and metabolic state to be maintained, so the determination of cytostatic autophagy is not unexpected. Although cytoprotective autophagy should also be considered as having the potential to inhibit cell growth, no studies have demonstrated that the protective autophagy that occurs after cell radiotherapy is actually growth arrest. However, it must be emphasized that the recently discovered cytostatic autophagy is quite different from cytoprotective autophagy. In A549 and H460 cells, Gewirtz and Kroemer Laboratories demonstrated that radiation alone produced cytoprotective autophagy and that radiation in combination with vitamin D or EB1089 could convert cytoprotective autophagy into cytostatic autophagy. The clinical significance of cytostatic autophagy is similar to that of cytotoxic autophagy; that is, if radiotherapy causes sustained growth arrest of tumour cells, inhibition of autophagy may reduce the effectiveness of the treatment.

We attribute cytotoxic autophagy and inhibitory autophagy to cellular non-protective autophagy. However, there is a need to consider the fact that there is currently no uniform method for detecting autophagy in clinical specimens, not to mention the definition of autophagy, and the results of current clinical trials are difficult to explain in the context of the basic concepts of different forms of autophagy. Autophagy in tumour cell fate is related to tumour type and the development stage, radiotherapy intensity, tumour cell tolerance and tumour microenvironment. We believe that the level of autophagy induced by different factors determines the ultimate fate of tumour cells. Autophagy induced by the tumour microenvironment may promote cell survival. In contrast, radiotherapy induces excessive or intense autophagy of tumour cells and may cause cells to 'eat themselves' and die.

25.2 The Effect of Autophagy on the Efficacy of Tumour Radiotherapy

25.2.1 Autophagy Enhances Radiosensitivity

PI3K/AKT/mTORC1 is an important signalling pathway for autophagy regulation, and inhibition of PI3K/AKT/mTORC1 can induce autophagy. NVP-BEZ235 is a novel PI3K/mTOR dual inhibitor. NVP-BEZ235 can effectively inhibit the PI3K/AKT/mTORC1 signalling pathway and increase the radiosensitivity of SU-2 glioma cells, PC-3 prostate cancer cells, DU145 and LNCaP cells. In these NVP-BEZ235-treated cell lines, more LC3 dot patterns were observed, and the level of membrane-bound LC3 II was significantly increased (Wang et al. 2013; Chang et al. 2014). Yu et al. (2012, 2015) reported that NU7441, a DNA-PK inhibitor, exerts radiosensitizing effects on C4-2 prostate cancer cells and A549 lung cancer cells by inducing autophagy. This effect may be due to increased endogenous expression of pS6K.

Chen et al. performed sirolimus treatment on glioma cell line SU-2, followed by X-ray irradiation. Compared with the direct X-ray irradiation group, the results showed that the cells in the irradiated group treated with the autophagy agonist had a lower autophagy rate and colony formation rate than those of the simple irradiation group, and the apoptosis rate was increased. These results indicate that glioma-initiating cells are more sensitive to X-ray irradiation after being treated with the autophagy agonist sirolimus. Kuwahara et al. (2011) studied the radiotherapy-resistant HepG2 cell line and showed that the autophagy-inducing agent rapamycin enhanced the radiation sensitivity of radiotherapy-resistant cells and inhibited the proliferation of tumour cells. The agent 3-MA enhances the radioresistance of the cells while reducing the expression of the Beclin-1 gene. Kim et al. (2006) showed that when bak/bak(-/-) breast cancer and lung cancer cells were irradiated, the expression of the autophagic death precursor protein complex was increased and radiation induced the expression of the Beclin-1 gene. The use of autophagy inhibitors reduces cell radiosensitivity. Thus, radioresistant tumour cells during radiotherapy can enhance the sensitivity of radiotherapy by increasing autophagy and induce the autophagic death of viable cells because autophagy can be destroyed under sustained and stable stress conditions. The structure of the cells, when it reaches a certain level, eventually leads to tumour cell apoptosis. Li et al. (2016) demonstrated that rapamycin-induced autophagy enhanced the radiosensitivity of the A549 lung cancer cell line, which was associated with prolonged phosphorylation of γ-H2AX and delayed radiation-induced repair of DSB.

An in vivo study has shown that radiotherapy-induced autophagy plays a crucial role in immunogenic cell death (ICD) (Golden et al. 2012). After radiotherapy, autophagy helps release cell death-related risk signals, such as calcium mesh eggs, HMGB1 and ATP released from dead cells, which represent the antitumour host immunity induced by chemotactic immune cells to tumour tissues that is required for the reaction. Chloroquine reduces ATP release and enhances the efficacy of radiotherapy in breast cancer by promoting immunogenic forms of cell death and better

antigen cross-presentation to increase endogenous radiosensitivity (Ratikan et al. 2013). Radiotherapy-induced autophagy plays a role in the clearance of tumours through the immune system and requires further investigation.

25.2.2 Autophagy Contributes to Radiation Resistance in Tumour Cells

Autophagy is closely related to tumour cell radiotherapy resistance. Numerous studies have shown that tumour resistance to radiation therapy is often associated with the upregulation of autophagy in a variety of tumour cell lines, such as colon cancer cells, prostate cancer cells, glioblastoma cells, nasopharyngeal carcinoma cells and breast cancer cells (Mo et al. 2014; Sun et al. 2015). A study by Lomonaco et al. (2009) showed that autophagy was induced by γ-irradiation of CD133+ glioma cells, and cell survival was decreased after 3-MA-mediated inhibition of autophagy. This radiotherapy-induced autophagy is protective, enhancing the resistance of CD133+ glioma cells to radiotherapy. Kim et al. showed that radiotherapy induced autophagy in non-small-cell lung cancer HCC827 cells and increased cell viability, but unexpectedly, although the autophagy induced by radiotherapy was protective, the Beclin-1-silenced cell line appeared to show increased radiotherapy sensitivity due to the increased cell survival and the downregulation of LC-3 II (Kim et al. 2013). The radiosensitivity of lung adenocarcinoma A549 cells was measured in normoxic and hypoxic cultures, and the results showed that the radiosensitivity of hypoxic A549 cells was decreased. Under anoxia, the autophagy-related genes HIF-1 and Beclin1 were downregulated, while the expression of HIF-1 and Beclin1 in hypoxic A549 cells was significantly increased, which induced autophagy, indicating that hypoxia-induced autophagy participates in the radiation resistance of lung adenocarcinoma. Chen et al. (2015) found that radiation-induced autophagy and radiotherapy resistance were inhibited in A549 lung cancer cells using radiotherapy in combination with shRNA-Nrf2 or the HO-1 inhibitor ZnPP.

The state of cellular stress caused by radiotherapy can affect the processing of proteins in tumour cells, so that the protein precursor cannot be correctly folded into functional proteins, resulting in a large amount of misfolded proteins accumulating in the lumen of the endoplasmic reticulum. To alleviate this endoplasmic reticulum stress state and survive, the cells must clear the misfolded protein that has accumulated in the lumen of the endoplasmic reticulum by autophagy, which is an important mechanism for tumour radiation resistance (Yu et al. 2011; Mizushima et al. 2008). On the other hand, radiation can cause DNA damage after acting on tumour cells and can induce autophagy after DNA damage. Autophagy, in this case, protects tumour cells because it reduces apoptosis induced by DNA damage (Polager et al. 2008; Abedin et al. 2007). In addition, DNA damage and functional disruption in the mitochondria can cause a large amount of ROS to damage cells, and autophagy can reduce the oxidative stress state of cells by eliminating functional mitochondria in

a timely manner, thereby mediating the resistance of tumour cells to radiation (Fan and Wang 2010). Therefore, in the process of radiotherapy, inhibiting autophagy in tumour cells in a certain way can initiate apoptosis-related signalling pathways to promote the apoptosis of tumour cells and improve therapeutic effects.

25.3 Autophagy Participates in DNA Damage and Post-injury Repair Caused by Radiotherapy

DNA is the main target of ionizing radiation in radiation therapy. Direct and indirect damage of ionizing radiation can cause DNA damage such as DNA double-strand breaks (DSBs), DNA single-strand breaks (SSBs) and base damage. Both normal cells and tumour cells have a complex set of mechanisms to deal with the occurrence of DNA damage to protect the stability and integrity of their genes. These damage repair mechanisms can be referred to as the DNA damage response (DDR). Autophagy and DNA damage and DDR processes involve many common genes and signalling pathways, and the relationships among them are very close. When radiotherapy is given to tumour cells, it causes DNA damage, cell cycle arrest and also induces autophagy. Autophagy has a complex dual role in DNA damage and DDR.

Autophagy has a large effect on the stability of DNA and can decrease DNA damage by controlling the quality of mitochondria and regulating ROS levels. Autophagy can also affect the DDR process by recovering the key proteins needed in the DDR process. In addition, autophagy provides a metabolic precursor for ATP production, which is required for multiple steps in the DDR process, while also regulating the dNTPs required for DNA synthesis during the DDR process.

Radiotherapy to tumour cells in a nutrient-deficient solid tumour can cause DNA damage. In the process of DDR, multiple steps are required to provide ATP, including helicase unwinding DNA during nucleotide excision repair, formation of the ATP-dependent chromosome recombination complex in DSB repair and PARP activation. Autophagy targeting glycogen, liposomes and proteins degrades these macromolecules and produces energy and metabolic precursors. Cells can synthesize ATP and other metabolic precursors promoted by autophagy for DDR that play very important roles in the DDR process. For example, treating glioma cells with TMZ and then administering autophagy inhibitors can inhibit the synthesis of ATP and increase mitotic mutations. If pyruvate is added, then the restoration of ATP levels will prevent mitotic mutations, indicating that autophagy maintains ATP production as a mechanism to promote gene stability.

dNTPs are an important element of DNA replication and repair. Autophagy can regulate dNTP reserve levels. Imbalances in dNTP levels increase gene mutations. Autophagy relies on maintaining balanced dNTP levels to counteract gene mutations, which is the basis for avoiding stress gene replication and amplification. Stress gene replication and amplification are usually observed in autophagy-deficient cells. For example, yeast cells treated with methyl methanesulfonic acid can produce autophagy

and promote the degradation of ribonucleotide reductase 1 (Rnr1). Rnr1 combines with other RNR proteins to deoxygenize ribonucleotides to deoxyribonucleotides. Rnr1 can optimize RNR activity and the dNTP levels utilized during DNA repair (e.g. MMR).

In addition to participating in the recovery of dNTPs and the production of ATP, autophagy is involved in the transformation of key proteins during DNA damage regulation. A recent study found associations among histone deacetylase (HDACS), a protein involved in DNA repair and apoptosis, DSB processes and autophagy. Treatment of cells with the HDAC inhibitor valproic acid can disrupt the activity of the Rad53 protein during double-stranded DNA repair. In valproic acid-treated cells, the Mre11 complex is recruited to the DSB injury site at the earliest time point and adheres to the DSB site with the development of the damage repair process, and the Mre11 complex is also responsible for the removal of Mre11 and the reduction of Sea2 levels from the lesion site during the DDR process. Inhibition of autophagy with the serine protease inhibitor PMSF or knockdown of the atg1 gene increases acetylated Sea2 levels. Conversely, induction of autophagy with rapamycin decreases acetylated Sea2 levels.

It was shown that rapamycin-induced autophagy damages the DSB repair process by degrading acetylated Sea2. These results show that, on the one hand, autophagy exerts a destabilizing effect, such as the acetylation of Sae2, which impairs DSB repair. On the other hand, autophagy clearance of Sae2 can also control cell damage caused by excessive DSB resection in DSB repair pathway. Xu et al. (Xu et al. 2017) showed that autophagy protects hematopoietic cells from radiation damage by promoting DNA damage repair. In this mechanism, autophagy promotes DSB repair in hematopoietic cells by inducing KAP1 degradation and activating STAT3 to upregulate BRCA1 expression, which has vital roles in DSB repair.

Autophagy can protect cells from death in the DNA damage response, but it can also play an opposite role in some cases and promote cell death. The dual role of autophagy in cell death can also be demonstrated via the regulation of autophagy by the MAPK pathway. Moderate activation of MEK/ERK can inhibit the mTOR pathway, slightly increase beclin1 activity and induce protective autophagy, while sustained activation of MEK/ERK leads to inhibition of the expression of mTORC1 and mTORC2, a sharp increase in beclin1 activity and induction of cytotoxic autophagy. Therefore, autophagy promotes cell death when severe DNA damage causes strong inhibition of mTORC1, accompanied by the activation of beclin1.

For the relationship between autophagy and DNA damage, it can be speculated that depending on the severity of the DNA damage or the type of damage, autophagy plays a different role: either to promote survival or to promote death. In this process, genes such as p53, p73 and E2F1 play important roles. With different degrees of DNA damage, they can not only promote DNA repair, cell cycle arrest or apoptosis but also regulate autophagy. It can be hypothesized that after minor DNA damage occurs, these transcription factors activate autophagy, leading to permeabilization of the mitochondrial membrane, production of dNTPs and/or ATP for DNA repair activities, degradation of pro-apoptotic proteins, activation of caspase8 and elimination of P62, thereby preventing overactivation of P38. For example, the p53 gene

mediates the transcription of the parkin protein, which can regulate the transcription of mitochondrial autophagy genes during DDR and promote autophagy and cell survival.

In contrast, autophagy also promotes the degradation of anti-apoptotic proteins, thereby promoting cell death. Studies have shown that in the late growth stage of Drosophila melanogaster, autophagy can mediate the degradation of the apoptosis inhibitor dBruce and degrade catalase in apoptosis-deficient cells, causing ROS accumulation and oxidative damage. These are the ways in which autophagy promotes cell death. As mentioned above, in valproate-treated yeast cells, autophagy can promote the degradation of acetylated Sae2, which can affect the damage repair of DSB in a more complicated way. By regulating certain enzymes involved in DNA damage repair, autophagy plays a role in promoting DNA damage persistence and enhancing apoptosis signals.

The intensity of autophagy activation and the target of autophagy are keys for determining whether autophagy promotes survival or death during DNA damage-induced cell death. Therefore, the relationship between DDR and autophagy is very important and can help us understand whether DDR-induced autophagy plays a positive or negative role in cell death.

25.4 Conclusion

Different forms of autophagy, cytoprotective autophagy and non-protective autophagy (including cytotoxic autophagy and cytostatic autophagy) occur in tumour cells in response to radiation. These forms of autophagy currently do not have clear morphological, biochemical or marker molecular differences. Additionally, there remains the problem that clinical data do not clearly confirm that any form of autophagy that occurs in tumour patients can be based on biopsy or circulating biomarkers. Given that the different forms of autophagy observed in experimental tumour cell lines are clinically relevant, the idea that autophagy inhibitors enhance radiosensitivity can only be established on the premise that radiotherapy-induced autophagy is cytoprotective autophagy. Another problem is whether the tolerable pharmacokinetics dose of these drugs is sufficient to promote autophagy in tumour cells in clinical trials using the autophagy inhibitor chloroquine or hydroxychloroquine. In addition, if a substance has been shown to be effective in inhibiting the autophagy of tumour cells, this is accompanied by autophagy interference of normal cells, making it impossible to maintain cell homeostasis and drug toxicity is also a serious problem. That is, the clinically minimal toxic doses of the antimalarial drugs chloroquine and hydroxychloroquine are precise because these drugs are not actually capable of inhibiting autophagy at these doses and are considered tolerable. Finally, there is no research on whether radiotherapy promotes autophagy (most likely a form of cytoprotection) in normal cells.

Some scholars have recently proposed the concept of the ‘autophagic switch’, which is different from the concept of enhancing or inhibiting autophagy. The

autophagic switch refers to turning cytoprotective autophagy into cytotoxic or cytostatic autophagy. The autophagic switch can also enhance the sensitivity of tumour cells to radiotherapy. Vitamin D and vitamin D analogues (EB1089) can sensitize breast tumour cells (ZR-75-1) and NSCLC cells to ionizing radiation through cytotoxic autophagy. Wilson et al. (2011) found that breast cancer cells (ZR-75-1) remained sensitive to radiotherapy when autophagy induced by the combination of radiotherapy and vitamin D or a vitamin D analogue was inhibited by drugs. This result indirectly shows that in the presence of vitamin D and vitamin D analogues, cytoprotective autophagy is turned into cytotoxic autophagy; in addition, even low levels of autophagy can maintain the radiosensitivity of tumour cells. However, the mechanism of the autophagic switch is unclear, which needs to be investigated.

Current clinical trials are based on insufficient evidence for autophagy induced by routine clinical treatment, even if autophagy is cytoprotective. In some cases, hydroxychloroquine may not be able to achieve sufficient intratumoural levels to inhibit autophagy and improve radiosensitivity. If studies in animal models indicate that activation of the immune response requires an autophagy signal, inhibition of autophagy may prove to be counterproductive. The result may be that rigorous research aiming to show that autophagy inhibition is truly a viable therapeutic strategy may be misunderstood due to the improper design of clinical trials. Conversely, if some trials prove to be successful, there may actually be different interpretations of the conclusions regarding the inhibition of protective autophagy. Chloroquine or hydroxychloroquine combined with radiotherapy may inhibit tumour growth more effectively than radiotherapy alone, but we cannot speculate whether this result is achieved by inhibiting autophagy. Therefore, the current trial results are related to the direct inhibition of protective autophagy, and whether they are successful or not, it is imperative to analyse whether autophagy is induced by radiotherapy or inhibited by chloroquine or hydroxychloroquine and whether it enhances therapeutic sensitivity.

Exploring autophagy as a new anticancer therapy is a promising research direction. Autophagy plays an important role in tumour suppression; at the same time, as a stress adaptive response, it can promote tumour cell survival and proliferation. In many tumour cells, autophagy prevents cell death caused by metabolic stress and an anoxic microenvironment. Autophagy regulation may become a new antitumour therapy associated with traditional cytotoxic drugs or targeted drugs. The relationship between autophagy and apoptosis and the relationship between autophagy and radiotherapy need further study. However, we believe that there will be more selective drugs for autophagy in the future, which will help humans finally cure tumours.

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Part IV
Autophagy and Immune Disorders

Autophagy is the protective cellular response to stress. It plays pivotal roles in the maintenance of cellular homeostasis by the degradation and recycling of cellular components, including proteins, amino acids, nucleic acids, and organelles. It is a fundamental eukaryotic process, which exists in various organisms such as plants, flies, mice, and humans. Despite its simplicity in single-cell eukaryotes, autophagy has been demonstrated to play a wide variety of physiological and pathophysiological roles with complex mechanisms. Autophagy has been suggested to be closely related to multiple diseases, especially immune disorders.

Immune disorders are diseases caused by dysfunction of the immune system, which can be characterized by the components of the immune system affected, overactive or underactive immune responses. Autophagy has been demonstrated to play critical roles in the regulation of the immune system, and therefore correlate with immune disorders. In recent years, the direct interaction between autophagy-related proteins and immune signals has been clarified by progress in genetics, biochemistry, cell biology, and systems biology. Autophagy could induce or inhibit immune reaction and inflammation under different conditions and it could be regulated by immune signals vice versa. Therefore, a complicated feedback network has been formed between autophagy and immunity.

Undoubtedly, autophagy plays a vital role in the regulation of immune reactions. For example, activation of the pattern recognition receptors (PRRs) could induce cellular autophagy which helps in the elimination of invading microorganisms. Moreover, besides affecting innate immunity through degrading endogenous inflammasome inducers and secreting inflammatory molecules, autophagy also regulates adaptive immunity via affecting antigen presentation and lymphocyte functions. Consequently, disorganized autophagy is closely related to several immune-related diseases. Based on our increasing understanding of the regulatory mechanisms of autophagy in diseases, autophagy is becoming the candidate target of therapies in immune-related diseases.

Chapter 26
Autophagy and Inflammatory Diseases

Min Jin and Yanyun Zhang

Abstract Autophagy is an essential biological process for cells to maintain their homeostasis. It is a complex regulatory system that integrates innate and adaptive immunity. The role of autophagy in immune diseases has been paid more and more attention with the deepening of the mutual regulation and mechanism of autophagy and immunity. It is found that the aberrant autophagy is closely related to inflammatory diseases, including infections, adaptive immune-associated inflammation and inflammatory bowel disease. Autophagy plays critical roles in the activation of NLRP3 inflammasome, the clearance of bacterial and viral infections and what is more, the function of adaptive immune cells.

Keywords Autophagy · Inflammasome · Infection · Antigen presentation · Inflammatory bowel disease

Autophagy is a fundamental cell biological pathway affecting immunity, which plays important roles in the identification and elimination of invading microbes, antigen presentation, and lymphocytes development. Innate immune responses such as antibacterial responses can activate autophagy. Meanwhile, autophagy regulates innate immune responses vice versa. They interact with each other to participate in multiple physiological and pathological processes.

26.1 Autophagy and Inflammation

In many cases, signals associated with inflammation can also regulate autophagy. The major receptors of PAMPs TLRs in macrophages and some other types of cells can regulate autophagy by activating downstream signaling. For example, CpG island which is the ligand of TLR9 can induce autophagy in rodent and human tumor cells, and the addition of bacterial LPS which is the ligand of TLR4 can also induce

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autophagy in macrophages. In recent studies, it has been shown that autophagy plays bipotential roles on inflammation. After myocardial ischemia–reperfusion (I/R) injury in autophagy-suppressed rats, the expression of IL-1 is decreased in the heart, indicating that autophagy aggravates the inflammatory response after cardiac I/R injury. Nevertheless, Atg16L1 knockout mice have increased the sensitivity to colitis and can be alleviated by IL-18 antibodies' treatment. Moreover, the activation of IL-1β and IL-18 in macrophages and monocytes have been enhanced in mice deficient in Beclin1 and LC3B, indicating that autophagy plays a negative role in inflammation under this condition.

The activation of cytokines in wild-type macrophages in response to LPS and ATP and the expression of cytokine in macrophages from in Beclin1- and LC3B-deficient mice are all involved in the signaling pathway of NLRP3 inflammasome. Autophagy deficiency can enhance the activation of NLRP3 inflammasome, which is regulated by mitochondrial homeostasis, including the increase of mitochondrial ROS and mitochondrial membrane permeability. Caspase-1-dependent IL-18 secretion in macrophages can be blocked by antioxidants targeting mitochondria. Consequently, autophagy can stabilize mitochondria and inhibit the activation of inflammasome. In general, it is suggested that autophagy plays distinct roles in inflammatory responses, which need to be further investigated in certain inflammatory diseases.

26.2 Autophagy and Pathogenic Infection

Macrophages/monocytes are the main type of innate immune cells, which play a major role in eliminating invading microbes. Activation of Toll-like receptors (TLRs) by microorganism exposure not only induces the production of inflammatory cytokines like IL-1β and IFNs but is also engaged in the induction of autophagy. It is found that zymosan particles (cell wall components of fungi) promote the fusion of LC3-positve autophagosomes with lysosomes via TLR2 signaling in macrophages. And in this process, Atg5 and Atg7 are required. Beyond that, TLR4 and TLR7 signaling also induces autophagy in macrophages/monocytes when stimulated with bacteria or bacterial components.

In addition to TLRs, NOD2 (nucleotide-binding oligomerization domain 2), an intracellular sensor recognizing muramyl dipeptide (MDP), can also induce the formation of autophagosome, and the subsequent promotion of antigen presentation in dendritic cells (DCs) stimulated with MDP (Cooney et al. 2010). Atg5, Atg7, and Atg16L1 are involved in this induction of autophagy. RIPK2 (receptor-interacting protein kinase 2), downstream regulator of NOD2 signaling, also participates in MDP-induced formation of autophagosomes. Nevertheless, NOD2 does not take a place in autophagosome formation induced by starvation or rapamycin treatment, indicating that NOD2 is specifically involved in bacterial-induced autophagosome formation.

Autophagy exhibits its anti-inflammatory capacity in the Crohn's disease mouse model with defective Atg16L1 which is an essential component of autophagy, showing increased interleukin-1β (IL-1β) and IL-18 production. Inflammasome is a multiprotein oligomer responsible for the activation of inflammatory responses, which promotes the maturation and secretion of pro-inflammatory cytokines IL-1β and IL-18 in respond to pathogen-associated molecular patterns (PAMPs) and endogenous damage-associated molecular patterns (DAMPs). The suppression of inflammasome activation by autophagy has been certified by many researches. Autophagy eliminates debris, aggregates, misfolded proteins, and defunct organelles which can act as endogenous agonists to activate inflammasome, so that the threshold of inflammasome activation is elevated due to the basal level of autophagy. Besides, autophagy downregulates inflammasome activation and inflammation by degrading aggregated inflammasome components or pro-IL-1-β (Harris et al. 2011). When autophagy is inhibited, depolarized mitochondria accumulate to leak endogenous inflammasome agonists like mitochondria DNA and reactive oxide species (ROS) and thus activates inflammasome. In conclusion, basal autophagy protects cells from inadvertent inflammasome activation, whereas deficient autophagy leads to enhanced inflammation via increasing inflammasome activation which results in a higher level of IL-1β. The interaction between autophagy and innate immunity is shown in Fig. 26.1.

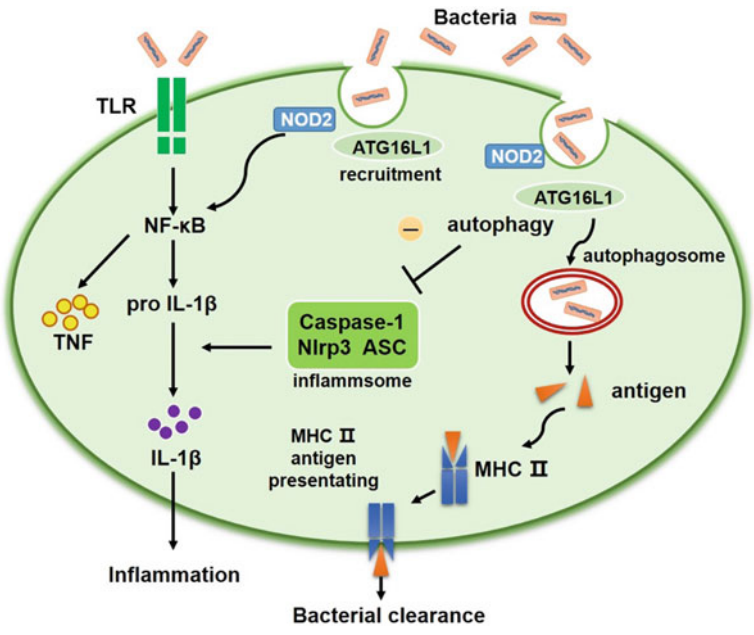

Fig. 26.1 The interaction between autophagy and innate immunity

Invading microorganisms can induce autophagy via innate immune receptors like TLRs and NODs. Meanwhile, invading microbes recognized by PRRs like TLRs are then taken up by phagocytosis and form autophagosomes. Even if a pathogen passes the autophagy barriers directed by conventional PRRs, it can still be captured in the cytosol by sequestosome-1-like receptors (SLRs) (Lee et al. 2007) or other mechanisms, including NOD2–autophagy-related protein 16 like 1 (Atg16L1) interactions, so-called xenophagy. SLRs contain cargo recognition domains (CDRs), which recognize ubiquitin or galectin on intracellular pathogens, and LC3-interacting regions (LIRs), which recruit membranes to autophagosomes. NOD2 initiates autophagic machinery by recruiting Atg16L1 to the plasma membrane at the site of bacterial entry. The vacuoles containing invaders, termed as autophagosomes, eventually fuse into autolysosomes for degradation and elimination. In the process of fusion, an autophagic process termed LC3-associated phagocytosis (LAP) plays a synergistic role to promote the maturation of autolysosomes. In summary, autophagy takes part in almost every stage of the elimination of microorganisms via phagocytosis (Fig. 26.2).

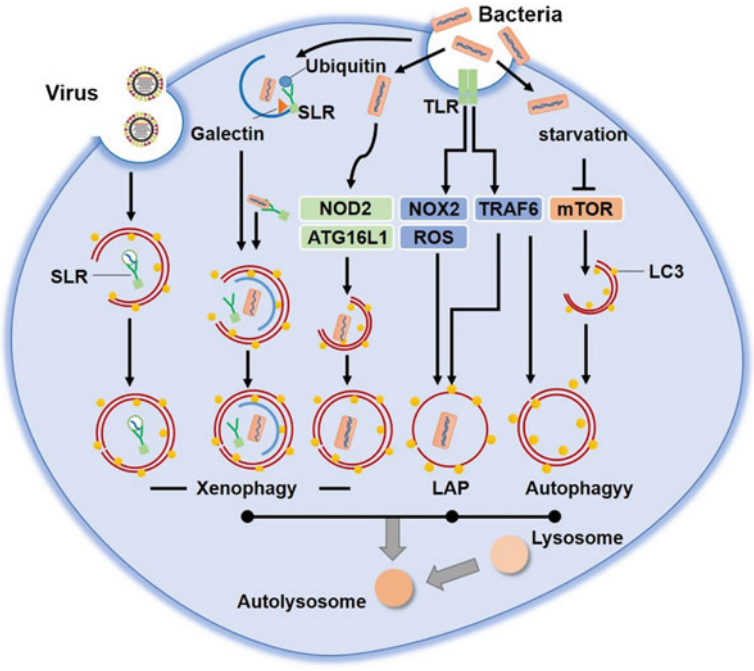

Fig. 26.2 Mechanisms of autophagy in eliminating microorganisms

26.3 Autophagy and Viral Infection

During virus infection, autophagy is induced in plasmacytoid dendritic cells (pDCs) to scoop the invading viral PAMPs and deliver them to the endosomal lumen where they contact with corresponding PRRs: including double-stranded (ds)RNA (TLR3), single-stranded (ss)RNA (TLR7 and TLR8), and DNA with unmethylated CpG sites (TLR9). The TLRs then recruit adaptors, such as myeloid differentiation primary response protein (MyD88) for TLR7/8/9 and TIR domain-containing adaptor molecule 1 (TRIF1) for TLR3/4. Both adaptors can activate NF-κB signaling to trigger type I IFN production, the main pro-inflammatory factors to kill virus (Lee and Kim 2007).

As for DNA virus, the genome of virus (mitochondrial or bacterial DNA) can induce the activation of stimulator of IFN genes protein (STING) through cGAMP synthase and cyclic GMP–AMP (cGAMP) production, which increases the type I IFN response. While autophagy acts as a negative regulator of this process by removing sources of agonists that stimulate STING. Moreover, autophagic factors (e.g., Atg9) inhibit the activation of STING by affecting its cytoplasmic translocation.

Besides, autophagy can degrade viral components, viral particles or even host factors required for viral replication, which is referred to as virophagy, to directly mediate the restriction of viral replication. For example, during Sindbis virus (SINV) infection, the virus capsid protein binds to P62, which interacts with LC3 through its LC3-interacting region (LIR) in help with E3 ubiquitin-protein ligase SMURF1 and Fanconi anemia group C protein (FANCC). This complex is packaged in autophagosome to be degraded through virophagy.

Autophagy mainly cooperates with immune response to be a part of antiviral response. However, during evolution, some viruses convert the autophagosome to their home for replication, such as foot-and-mouth disease virus. On the one hand, the autophagosome provides viruses a membrane-bound, protected environment to generate their progeny, where autophagy-generated metabolites and energy are utilized for virus replication (O'Donnell et al. 2011). On the other hand, viruses can also hijack lipophagy, another type of autophagy degrading lipid droplet in cells. Since lipid droplets are an ideal platform for virion assembly, viruses can maintain the high level of ATP required for viral replication by directly activating lipophagy (Samsa et al. 2009).

Overall, innate immune responses induced by invading microorganisms or accumulated damaged autologous molecules can activate autophagy. Furthermore, autophagy plays distinct roles in innate immune responses under different circumstances. Autophagy suppresses the activation of inflammasome to inhibit the relevant immune response, as well as promotes the innate immune response to eliminate invading microbes. Reasonable application of interventional means of autophagy to the therapy of autophagy-dependent immune-related diseases such as Crohn's disease will be promising.

26.4 Autophagy and Adaptive Immune-Mediated Inflammation

Autophagy not only can remove unnecessary intracellular substances (such as damaged organelles, redundant proteins), but also can be used to activate the immune system, including adaptive immunity. The function of autophagy-related proteins in adaptive immunity mainly focuses on the presentation of antigen and the development and homeostasis of immune system. Autophagy promotes the activation of adaptive immune cells such as $CD4^+$ T cells and $CD8^+$ T cells, and improves the antigen presentation of antigen-presenting cells (APCs) to initiate adaptive immune response.

The immune response mediated by T lymphocytes firstly depends on the identification of antigen, which subsequently induces the activation and proliferation of T lymphocytes and their function by activating TCR. According to the present study, autophagy has important regulatory roles in the process of APC presenting antigen (Munz 2016). APCs are responsible for the processing and presenting of antigens and the formation of antigen polypeptide–MHC complexes presented to T cells, and thus activating T cells. According to the origin of antigen, the way of antigen presentation can be divided into endogenous antigen presented to $CD8^+$ T cells by MHC I molecular pathway, exogenous antigen presented to $CD4^+$ T cells through MHC II molecular pathway and cross-presentation pathway.

The occurrence of autophagy in APCs can improve the ability of MHC I and MHC II molecular pathways to present antigens. It is found that inhibiting macrophage autophagy would weaken the presentation of exogenous complement 5 (C5) while inhibiting DC autophagy impairs its ability of presenting endogenous mucin 1 (MUC1) via MHC II pathway. The autophagy-related proteins are also necessary for cross-presentation after infection. Cross-presentation is a nonclassical antigen presentation pathway, which means APCs can either present extracellular antigens degraded by lysosome through MHC I molecules or present endogenous antigens through MHC II molecules. The absence of autophagy-related proteins Atg12 or Beclin1 almost completely abolished APCs' cross-presentation of tumor antigens, while rapamycin or starvation-induced autophagy enhanced this ability (Nakatogawa et al. 2007). Thus, the occurrence of autophagy in APCs can enhance antigen presentation and promote T cells activation (Fig. 26.3).

Autophagy affects adaptive immune responses by regulating T cell development, differentiation, maturation, and homeostasis. Firstly, the removal of mitochondria by autophagy is conducive to the self-renewal of hematopoietic stem cells, which is critical for the production of lymphoid and myeloid progenitors (Mortensen et al. 2011). Secondly, the elimination of self-reactive T cells by autophagy during the development of T cell in thymus is pivotal to guarantee the development and homeostasis of the immune system. During T cells development from thymus lymphoid stem cells, the number of mitochondria is strictly regulated. If mitochondria cannot be effectively removed due to autophagy deficiency, there will be developmental

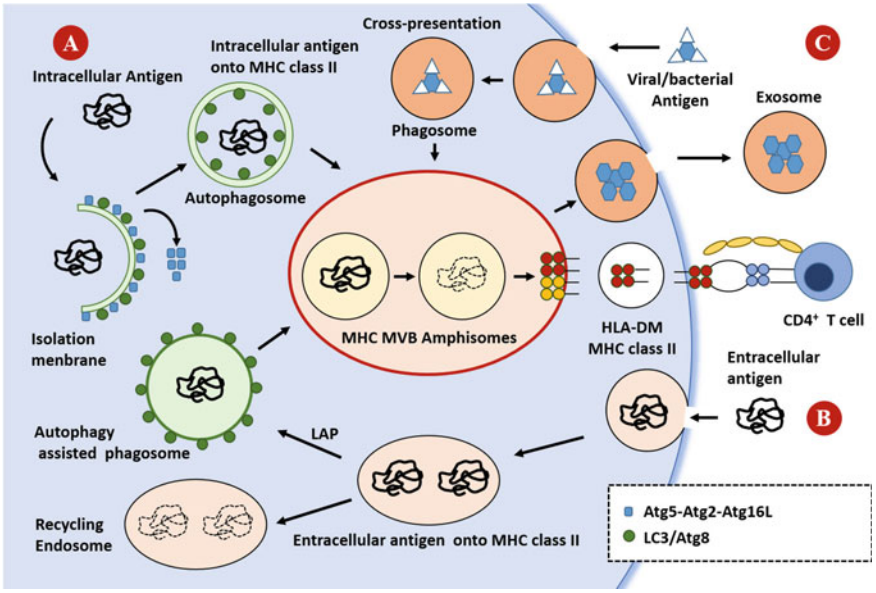

Fig. 26.3 The role of autophagy in antigen presentation

defects in T cells. Even after leaving the thymus, the development and maturation of T cells still largely depends on autophagy.

Additionally, autophagy in T cells will affect its own function. When $CD4^+$ T cells are activated, the expression of intracellular autophagy-related proteins, such as Atg5, Atg7, Beclin1, and LC3 will be upregulated, indicating that the activation of $CD4^+$ T cells could induce autophagy (Jacquin and Apetoh 2018). In Atg7-deficient T cells, endoplasmic reticulum accumulates in the cytoplasm, resulting in the accumulation of calcium within cells which limits the normal flow of calcium in TCR response and impairs T cells activation. In naive T cells, the cellular FLICE-like inhibitory protein (cFLIP) inhibits the initiation of autophagy, while the TCR signal and CD28 co-stimulation induce autophagy in activated T cells. Further exploration found that this process is independent of mTOR signal. Autophagy is induced in activated $CD4^+$ T cells by autocrine and paracrine IL-2 and IL-4 through JAK/STAT signaling pathways. Autophagy can promote the proliferation of $CD4^+$ T cells and the production of cytokines, and also regulate the differentiation of $CD4^+$ T cell. For example, although $CD4^+$ T cells with the absence of Atg5 display reduced survival, the production of IL-9 is upregulated to promote the differentiation into Th9 cells, which possess antitumor efficacy. Meanwhile, autophagy can also affect the survival, proliferation and differentiation of $CD8^+$ T cells. After Atg5 knockout, the survival and proliferation of $CD8^+$ T cells are impaired, accompanied by the decreased proportion of memory T cells. Other studies have shown that autophagy promotes the CTL function of $CD8^+$ T cell by improving the ability of MHC II molecular antigen presentation (Perot et al. 2013).

Autophagy can also regulate the adaptive immune response by affecting the development and function of B cells. In Atg5 knockout mice, the differentiation of progenitor B cells to pre-B cells and immature B cells is impaired, resulting in defected B cell development (Miller et al. 2008). After activated by antigen, mature B cells further differentiate into plasma cells and memory B cells. Plasma cells mainly produce antibody scavenging antigen while memory B cells can be reactivated and differentiate into plasma cells when encountering the same antigen, which speed up the process of humoral immune response. Autophagy is essential for the homeostasis of plasma cells and the survival of memory B cells. It is found that in the bone marrow of autophagy-deficient mice, the proportion of long-lived plasma cells decreased significantly compared with that of normal mice. Knockout of autophagy-related genes in B cells will further activate endoplasmic reticulum stress signal, leading to the death of plasma cells (Pengo et al. 2013). In addition, the production of antibodies in mice with autophagy-deficient B cells infected with influenza virus twice is inhibited. Therefore, autophagy plays an important role in B cell development, plasma cell survival, and antibody production.

In short, autophagy can participate in adaptive immunity by regulating antigen presentation, T cell homeostasis, and the development and function of B cells. Therefore, it can participate in the occurrence and development of inflammatory diseases by regulating the function of immune cells and related immune responses.

26.5 Autophagy and Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a group of chronic and nonspecific intestinal inflammatory diseases whose etiology and pathogenesis are not completely clear. It is characterized by chronic gastrointestinal inflammation. Ulcerative colitis (UC) and Crohn's disease (CD) are the two major types of IBD. The main difference between CD and UC lies in the location of inflammation and the inflammatory reaction itself. CD can affect any part of the digestive system and cause leaping lesions, mostly at the end of the ileum, and with transmural injury. However, UC is limited to the mucous membrane of the colon and rectum. IBD is caused by the interaction of many factors, such as intestinal flora imbalance, oxidative stress, and immune abnormality, which leads to intestinal mucosal injury and ulceration and eventually to the disease. IBD is closely related to the change of mucosal barrier function.

Genome-wide association studies have linked autophagy-related gene Atg16L1 polymorphism with the susceptibility to IBD. This section takes Crohn's disease as an example to discuss the role of autophagy in the pathogenesis of IBD. Crohn's disease is characterized by inflammation, ulceration, and granulocyte entry in the small intestinal epithelium. The pathogenesis of Crohn's disease may be related to intestinal flora imbalance, excessive inflammatory response, and abnormal autophagy. NOD2 is the first recognized gene related to Crohn's disease. It is a member of the NLR family whose leucine repeat sequence of the carboxyl terminus (LRR) can recognize the MDP of bacteria. The activation of NF-κB can be mediated by a series of

signaling molecules (including RIPK2 and CARD9) and induces the expression of pro-inflammatory cytokines (including IL-6, TNF-α, and IL-1β). Studies have found that the intestinal dominant bacteria in NOD2 knockout mice decrease significantly, indicating that NOD2 may reduce the risk of Crohn's disease by maintaining the number of dominant bacteria. Autophagy can be induced after NOD2 activated by MDP, to promote the binding of antigenic peptides and MHC-II molecules in dendritic cells and eventually eliminate intracellular bacteria. Similar to NOD2 deficiency, the mutation of NPC1 can result in impaired autophagy, but it will not impair the production of NOD2 receptor interaction kinase 2 (RIPK2)-XIAP dependent cytokines. Activation of autophagy can increase autophagy flux and thus bypass NPC1 defect to restore the bacterial clearance ability of macrophages.

In addition to intestinal microbial ecological imbalance, intestinal mucosal barrier dysfunction can also be caused by endoplasmic reticulum stress (ER stress) of intestinal epithelial cells. Multiple stimuli (such as nutrient substrate deficiency, pathogenic microorganisms, inflammatory factors, etc.) can cause the accumulation of unfolded or misfolded proteins in the endoplasmic reticulum and induce ER stress. ER stress will cause endoplasmic reticulum dysfunction and activate unfolded protein response (UPR). The homeostasis of endoplasmic reticulum is maintained by decreasing the synthesis of new proteins and increasing the synthesis of chaperones and the degradation of misfolded or unfolded proteins. Recent studies suggest that ER stress is the inducer of autophagy. When UPR is activated by ER stress, it can induce autophagy to clear misfolded or unfolded proteins and to balance the expansion of endoplasmic reticulum, avoid apoptosis, and improve cellular survival. However, the mechanism of ER stress-induced autophagy is not clear yet.

A large number of lymphocytes have been found in the lamina propria of intestinal mucosa in IBD patients. Under normal conditions, the dynamics between Th1 and Th2 cells determines the balance between pro-inflammatory and anti-inflammatory cytokines. But when the body is attacked by antigen, this balance is broken, leading to the occurrence of inflammation. Many autophagy-related proteins (such as Beclin 1, Atg3, Atg5, and Atg7) are necessary for the development, maturation, and survival of T cells. When autophagy is abnormal, the proportion of Th1/Th2 cells is out of order and the balance between pro-inflammatory and anti-inflammatory cytokines is disturbed, leading to severe inflammation in the gut and eventually IBD.

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Chapter 27
Autophagy and Immune-Related Diseases

Min Jin and Yanyun Zhang

Abstract As a result of its multifunction both in innate and adaptive immune systems, autophagy has been demonstrated to take part in the pathogenesis of several immune-related diseases. The study on the pathological mechanism of autophagy in these diseases may provide an experimental and theoretical basis for targeted intervention of autophagy in the prevention and treatment of immune diseases. To date, it has been reported that autophagy can eliminate impaired mitochondrial to inhibit the activation of NLRP3 inflammasome which promotes the progression of atherosclerosis. Moreover, enhanced autophagy can effectively prevent the occurrence of GVHD. It also plays a key role in the development of viral hepatitis. Therefore, autophagy might be a promising regulatory target for the treatment of immune-related diseases.

Keywords Autophagy · Atherosclerosis · Graft-versus-host disease · Inflammatory liver disease

27.1 Autophagy and Atherosclerosis

Autophagy is an essential process to maintain cell homeostasis which is important for cell survival, differentiation, and development. Increasing evidences suggest that it has become a therapeutic target for multiple diseases such as inflammatory diseases, cardiovascular disease, autoimmune disease, nervous system diseases, and cancers.

Atherosclerosis is a pathological process in which lipid and fibrous components are deposited in the arterial wall. During the process of disease, mononuclear cells in the blood adhere to the endothelium and migrate to the subcutaneous. Lipoprotein particles deposited under the intima are easily oxidized, which will induce local inflammatory reaction and the differentiation of monocytes into macrophages followed by the transformation into foam cells. Foam cells aggregate to form lipid streaks, while smooth muscle cells migrate to the inner membrane, producing an extracellular matrix to form a plaque that encapsulates the lipid core with the fibrous

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cap. Atherosclerosis is the most common cause of acute myocardial infarction and coronary heart disease. Currently, 3 million people die of cardiovascular disease each year in China.

Long-term studies have shown that the process of atherosclerosis is the result of a combination of many factors with the infiltration of a variety of inflammatory cells, indicating the inflammatory response a key factor in the occurrence of the disease. The mouse model of atherosclerosis confirms that mTOR inhibitors, which can also induce autophagy, can reduce the cholesterol content in the aortic arch to delay the development of atherosclerosis. The use of TLR7 ligand imiquimod can induce VCAM-1 and T lymphocyte infiltration, macrophage accumulation, and plaque enlargement by inducing the release of proinflammatory chemokine through autophagy induced in macrophages. NLRP3 inflammasome promotes the progression of atherosclerosis by enhancing the release of IL-1β, suggesting that NLPR3 is a potential therapeutic target for atherosclerosis. Studies have shown that autophagy can eliminate impaired mitochondrial to inhibit the activation of NLRP3 inflammasome activated by mitochondrial reactive oxygen species (ROS) (Zhou et al. 2011). Furthermore, autophagy can also regulate the activity of inflammasome by the capture and degradation of NLRP3 inflammasome. Above all, studies provide a theoretical basis and potential ideas for the prevention and treatment of atherosclerotic lesions by regulating autophagy.

27.2 Autophagy and Graft-Versus-Host Disease

Graft-versus-host disease (GVHD) is a posttransplant immune rejection induced by mismatched histocompatibility antigens during bone marrow/hematopoietic stem cell transplantation, which often leads to transplantation failure and transplantation related death. Rapamycin is a commonly used immunosuppressant in clinic. It is often used to inhibit immune rejection after bone marrow/hematopoietic stem cell transplantation and is also one of the main autophagy inducers. Animal experiments and clinical trials show that rapamycin can effectively prevent the occurrence of GVHD and alleviate its symptoms. Recent studies on autophagy and GVHD suggest that autophagy may be involved in this process. It has been found that enhanced autophagy can effectively prevent the occurrence of GVHD, while and the deletion of autophagy-related gene Atg16L1T and Atg16L1T^{T300A} polymorphism can increase the mortality after allogeneic hematopoietic stem cell transplantation. The expression of inhibitory molecule A20 of DC activation is upregulated, while the lysosomal activity is suppressed in DCs with Atg16L1T deletion, accompanied by upregulated NF-κB and MAPK signaling pathway and promoted costimulatory molecule expression on DC surface. Therefore, by upregulating the activity of DCs, autophagy can promote the induction and activation of T cells in recipients, thus promoting the proliferation and activation of allogeneic T cells and exacerbating the immune rejection of recipient tissues and organs (Hubbard-Lucey et al. 2014).

27.3 Autophagy and Inflammatory Liver Diseases

In inflammatory liver diseases, the decrease of autophagy will aggravate inflammation and lead to liver fibrosis and even liver cancer. When the autophagy of Kupffer cells in the liver is inhibited, the damaged mitochondria could not be cleared in time. The excessive accumulation of ROS further activates NLRP3 inflammasome and causes an excessive inflammatory response. It has been found that induced autophagy can effectively alleviate acute liver injury by inhibiting NLRP3 inflammasome in mice (Han et al. 2016). However, autophagy functions differently in viral hepatitis. Replication of hepatitis B virus (HBV) or hepatitis C virus (HCV) depends on autophagy. Taking HCV as an example, when liver cells infect HCV genome, autophagy formation increases, while autophagy degradation decreases due to the inability to form mature lysosomes. On the one hand, autophagy-related genes, such as Atg5, Atg7, Atg12, and Beclin1, can initiate the replication of HCV. On the other hand, the RNA polymerase (NS5A, NS5B) and HCV RNA aggregated on autophagy also provide sufficient raw materials for virus replication and packaging (Tanida et al. 2009). Clinical data show that the number of autophagosomes in liver cells from HCV patients is significantly higher than that from healthy people, which further indicates that autophagy plays a key role in the development of viral hepatitis. It also suggests its potential effect on the treatment of such diseases (Rautou et al. 2011).

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Chapter 28
Autophagy and Autoimmune Diseases

Min Jin and Yanyun Zhang

Abstract Alterations of autophagy contribute to the progression of various autoimmune diseases, including systemic lupus erythematosus (SLE), inflammatory bowel disease (IBD), rheumatoid arthritis (RA), and systemic sclerosis (SSc). In patients with SLE, autophagy defects result in poor clearance of phagocytic fragments and excessive secretion of inflammatory factors. The disorder of autophagy in IBD patients is closely related to the regulation of inflammatory factors and the clearance of pathogenic pathogens of enteropathy. The increase of autophagy in synovioblasts of RA patients will promote RA-associated synovitis. The autophagy of fibroblasts in SSc patients is dysfunctional, leading to overactive wound healing. Understanding the role of autophagy in the pathogenesis may give us hints on the therapy of autoimmune diseases.

Keywords Autophagy · Systemic lupus erythematosus · Rheumatoid arthritis · Systemic sclerosis

Immune tolerance is an important part of immune homeostasis. If the immune system loses tolerance to its own tissues, it will lead to various immune-related diseases, such as autoimmune diseases, allergic reactions, and transplant rejection. Autoimmune diseases are caused by the excessive activation of autoreactive T and B cells, the production of large numbers of autoantibodies, and the inability of the immune system to distinguish between self and nonself when self-tolerance breaks down. The autoimmune system produces an immune response to its own cells and tissues, leading to tissue damage and organ dysfunction. Autophagy, an endogenous process necessary for the turnover of organelles, maintains cellular homeostasis, and directs cell fate.

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28.1 Autophagy and Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a heterogeneous autoimmune disease, defined by loss of B cell self-tolerance that results in the production of antinuclear antibodies (ANA) and chronic inflammation. Defects in the clearance of dying cells have been proposed to underlie the pathogenesis of SLE. Apoptosis plays an important role in the development and immunomodulation of lymphocytes. Apoptotic cells can be regarded as a huge store of autoantigen and the accumulation of apoptotic bodies leads to the increase of their own antigens, which may promote the production of autoantibodies. Studies have shown that apoptosis of lymphocytes, neutrophils, monocytes, and macrophages in SLE patients increased significantly. The increase of macrophage apoptosis may lead to leakage of antigen and hampered clearance of other apoptotic bodies, which may promote the production of autoantibodies. In addition, components released by macrophages and other cells after death may accelerate the maturation of dendritic cells and then cross-presenting the antigens of these apoptotic cells to cytotoxic T cells. Simultaneously, autoreactive T cells in lymph nodes are activated to release cytokines such as IL-1 and TNF-α to establish the inflammatory microenvironment and induce an excessive autoimmune response. The pathogenesis of SLE may be related to the dysfunction of apoptotic bodies' clearance. The nucleosome produced by apoptosis is the main autoantigen of SLE, which is highly specific to SLE.

There is a close relationship between autophagy and apoptosis. Autophagy can either promote apoptosis or inhibit this process to maintain cellular homeostasis. Autophagy-induced apoptosis, i.e., type II-programmed cell death, is characterized by the appearance of autophagosomes and independent of caspase. Autophagosomes can digest the contents by fusing with lysosomes. Studies suggest that autophagy-related proteins can degrade pro-apoptosis proteins, such as caspases, to inhibit cellular apoptosis. Rapid clearance of apoptotic cells is critical to prevent inflammation. Severe inflammation exists in autophagy-deficient fetus ($Atg5^{-/-}$), accompanied by defective clearance of apoptotic cells. Meanwhile, defect in a noncanonical autophagy, known as LC3-associated phagocytosis (LAP), results in impaired digestion of engulfed dying cells and leads to increased inflammatory cytokine production and SLE-like disease in mice (Allison 2016). Meanwhile, the transmembrane potential of T cell in SLE patients increases, leading to mitochondrial hyperpolarization and ROS accumulation which finally results in apoptosis and necrosis of T cells. Overall, the abnormal mitochondria of T cells may also be one of the mechanisms of the increased apoptosis in SLE patients. Morphologic analysis of T cells from SLE patients suggests the existence of large mitochondria, which might be due to the defect of mitochondrial-specific autophagy.

In addition, GAWS and meta-analysis show that gene polymorphisms of Atg5, DRAM1, and IRGM are associated with SLE. Atg5, DRAM1, and IRGM are autophagy-related proteins, among which DRAM1 is the important downstream molecule of p53, encoding a lysosomal membrane protein regulating autophagy

and apoptosis. Moreover, the activation of mTOR signaling might be involved in the dysfunction of T cells and the pathogenesis of SLE as well.

28.2 Autophagy and Rheumatoid Arthritis

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation of the synovium associated with apoptosis and destruction of the joint. The proliferation of synovioblasts in RA patients is upregulated due to the decrease of apoptosis. In joint synovioblasts of RA patients, a variety of mechanisms endow the cells with the ability to resist programmed cell death, including increased levels of anti-apoptotic factors, downregulation of pro-apoptotic factors, and promoted autophagy.

The expression of citrulline is upregulated in synovioblasts and synovial fluid of RA patients. APCs play an important role in the activation of autoreactive T cells while autophagy can regulate the expression and of citrullination of antigen through APCs. Meanwhile, autophagy induces the expression of citrulline antigen in B cells (with the help of B cell receptors), resulting in the activation of T cells involved in the development and progression of RA (Weindel et al. 2015). It is found that the expression of Atg7/Beclin-1 and autophagy are increased in the osteoclasts of RA patients, and the monocytes are induced to differentiate into mature osteoclasts, which increase the ability of bone resorption of osteoblasts. In conclusion, the increase of synovioblasts autophagy, the activation of T cells by autophagy, and the dysfunction of autophagy in osteoclasts and osteoblasts are all the key factors to change the fate and function of cells in RA. It is suggested that autophagy plays a key role in joint destruction and regulating autophagy may be a method to prevent bone resorption in the treatment of RA.

28.3 Autophagy and Systemic Sclerosis

Systemic sclerosis (SSc) is an autoimmune disease characterized by increased collagen deposition and fibrosis and excessive wound healing caused by the persistence of fibroblasts. Pathology can be confined to the skin of the face and limbs and can also affect internal organs. Dysfunctional autophagy is thought to play a key role in SSc (Rockel and Kapoor 2016). In normal renal fibroblasts, TGF-β activates autophagy by acting on Beclin1 and promotes the degradation of type I collagen to regulate collagen deposition. Plasma membrane microcapsule protein-1 is a receptor for circulating TGF-β in cells. Its reduction can activate TGF-β signal. In SSc patients, the decrease of plasma membrane microcapsule protein-1 and the activation of TGF-β signal cause autophagy abnormality, collagen deposition and inhibition of myofibroblast differentiation. Autophagosomes have been found in most of the skin biopsy specimens (>87%) of SSc patients, i.e. increased LC3 staining. However, the autophagy flux is

decreased, indicating that the degradation of intracellular components mediated by autophagy is blocked. This suggests that upregulation of autophagy may serve as a potential therapy for SSc.

28.4 Conclusion

Autophagy-related proteins are involved in biological processes ranging from the onset of autophagy to the degradation of the contents, playing an important role in the regulation of immunity and inflammation, and participate in the occurrence of immune-related diseases. Autophagy can also be involved in infectious, inflammatory, and autoimmune diseases through numerous molecular mechanisms. The deep and systematic understanding of the interaction between autophagy and immunity will not only help us understanding the mechanism of immune-related diseases, but also greatly promote the exploration and application of the intervention and treatment of immune-related diseases.

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Part V
Autophagy and Infection

Thirty years ago, people have observed the phenomenon of infection-causing autophagy. Autophagy plays an important role in the infection of a variety of pathogens, including bacteria, viruses, fungi, parasites, and the like. Autophagy can play an anti-infective role by directly removing intracellular microorganisms (bacteria, viruses, parasites, etc.) and toxins (*S. aureus* α-toxin, etc.); it can also indirectly regulate the host immune responses by eliminating the damage-related molecular patterns (DAMPs) or degradation of inflammatory bodies, clearing molecules on the corresponding signaling pathways, and inhibiting type I interferon (IFN) responses. In the long-term evolution of microorganisms, microbes have also developed a variety of strategies to resist and utilize autophagy, which is more conducive to their own survival.

Direct removal of pathogenic microorganisms (heterogeneous phagocytosis) by autophagy is the most important autophagy pathway in pathogen infection. Whether it is noninfectious or infection-related autophagy, it plays an equally important anti-inflammatory role in protecting the host. Noninfectious autophagy can use the invading microorganisms such as bacteria or viruses as a substrate for autophagy. It mainly includes Mycobacterium tuberculosis which infects macrophages or animals, and Group A *streptococci* which invade host cells. Many other bacteria, including *Listeria*, *Salmonella,* and *Shigella*, have been shown to be sensitive to autophagy in cellular experiments. Similarly, viruses and protozoa can be directly or modified to become autophagic substrates.

The host cell recognizes the cytoplasmic pathogen and subsequently delivers the pathogen to the lysosome by an autophagy process. Autophagy can sequester the cytoplasm to be degraded in the newly formed membrane-bound region (i.e., autophagosome). Autophagosomes are formed by the growth of crescent-shaped membrane structures (phagocytic cells) and eventually surrounding the cytosol. At the time of infection, eukaryotic cells can use selective autophagy to protect the cytoplasm against bacterial invaders, attack vacuoles containing microbes, and remove inclusion bodies from pathogens. Selective autophagy is dependent on autophagy receptors, such as the autophagy associated gene (ATG) 8 family of ubiquitin-like

proteins expressed on the phagocytic membrane that can be cross-linked to the "self-clearing" signal of the substrate. Heterologous phages have many identical self-clearing signals, which allow autophagy to phagocytize host protein polymers and damaged organelles. At the same time, the autophagy protein can also directly act on microbial molecules, such as ATG5, which can directly bind to the surface protein VirG of *Shigella*. In *Toxoplasma*-infected macrophages, ATG5 recruits immune-associated GTPase-mediated destruction of parasite vesicles, leading to parasite death.

Under strong evolutionary selective pressure, the host's autophagic ability to microorganisms can be eliminated by bacterial or viral adaptation, and most of the pathogens that successfully escape autophagy have their own unique anti-autophagy strategy: inhibition of autophagy initiation, the maturation of autophagosomes, and the recognition of autophagy, and even the use of autophagy components to promote their own replication and survival. A variety of pathogens can be adapted, including *Shigella*, *Legionella*, *Mycobacterium tuberculosis*, herpes simplex virus 1 (HSV-1), and human immunodeficiency virus (HIV). As with other host-pathogen interactions, the balance between microorganisms and hosts can lead to the development of chronic diseases, subclinical diseases or latent infections, such as *tuberculosis* latency or persistent viral infections. Some bacteria can actively evade autophagy. For example, *Shigella* and *Listeria* can escape from the vacuole to the cytosol after internalization, and actively avoid autophagy degradation. Some bacteria use host autophagosomes for replication, such as *Staphylococcus aureus*, phagocytic anaplasma, *Yersinia pseudotuberculosis*, and *Yersinia pestis*. In this case, inhibition of autophagy is not conducive to bacterial replication, while induction of autophagy promotes bacterial infection. There are also mechanisms for antagonizing autophagy in the virus, such as herpesvirus and HIV-encoded proteins that directly inhibit autophagosome maturation by inhibiting the core autophagy protein Beclin-1.

Bacteria are the first creatures on Earth, almost ubiquitous. Bacterial infection is a common clinical disease that can affect a variety of organs and tissues. Such as *Escherichia coli*, *Salmonella typhi*, *Salmonella,* and *Shigella* can cause bacterial enteritis. *Mycobacterium tuberculosis* can invade susceptible organisms through the respiratory tract, digestive tract, or skin lesions, causing tuberculosis in a variety of tissues and organs, among which *tuberculosis* is the most common through the respiratory tract. After infection with *Listeria monocytogenes*, it mainly manifests as sepsis, meningitis, and mononucleosis. *Helicobacter pylori* is not only associated with chronic gastritis, peptic ulcer, and gastric cancer, but also causes other organ and tissue diseases such as cardiovascular disease, anemia, and idiopathic thrombocytopenic purpura. Multicellular organisms live in the oceans of bacteria and respond to bacteria through innate and adaptive immunity. Although the nature of autophagy is the degradation of cells by their own substances, it has a wide interaction with pathogens in innate and adaptive immunity. The role of autophagy is

a “double-edged sword”: on the one hand, pathogens invasion can induce the formation of autophagy, thereby degrading the pathogen and protecting the cells from damage. On the other hand, bacteria have evolved a unique mechanism that hinders autophagy-mediated transport of pathogens to lysosomes, preventing their subsequent degradation by lysosomes and facilitating their own replication and survival.

Chapter 29
Autophagy and Bacterial Infection

Yichuan Xiao and Wei Cai

Abstract Bacterial infection is a common clinical disease that can affect a variety of organs and tissues. Autophagy, as an important part of the innate immune response and adaptive immune response, plays an important role in the defense against bacterial infection. Bacteria can also evade autophagy by destroying or utilizing autophagy virulence proteins or related molecules. Studying the mechanism of autophagy in bacteria and its interaction with cells help to discover new pathogenic mechanisms of bacterial infection. This chapter introduces the possible mechanisms of autophagy during bacterial infections such as *Salmonella* and *Mycobacterium tuberculosis*, in order to discover new ways to prevent and control infectious diseases.

Keywords Autophagy · Bacterial infection · Immune · Interaction

29.1 Regulation of Bacterial Infection by Autophagy

Autophagy effectively limits the bacterial subpopulations that inhabit cell vesicles and exposes them to the cytosol by destroying their restricted vacuolar membranes, such as *Mycobacterium tuberculosis* and *Salmonella typhimurium*. And specialized cytoplasmic pathogens such as Listeria can avoid this type of attack. The phagocytic recruitment of bacteria depends on three autophagy receptors, nuclear dot protein 52 (NDP52), p62 and optinin (OPTN). Their bacterial-associated self-clearing signals are derived from (1) ubiquitin-protein deposition activated by host cells surrounding susceptible bacteria, such as the E3 ligase LRSAM1; (2) bacterial damage to the vacuolar membrane, which exposes the polysaccharide to the cytosol, via galactose agglutination-8 binds to and recruits NDP52 (3) to release mycobacterial DNA after

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phagocytic membrane permeabilization through the type VII secretion system ESX-1, which is recognized by the stimulator of interferon genes (STING) and causes pan Prime deposition.

In addition to chelation with cytosolic pathogens, autophagy receptors also select cytoplasmic proteins for autophagy, and antibacterial peptides produced by cytoplasmic protein digestion contribute to the bactericidal properties of autophagosomes. IFN-induced 65-kD guanine nucleotide-binding regulatory proteins (GBPs) facilitate the transport of p62-bound cytoplasmic proteins to autophagic vacuoles, resulting in the production of bacterial lytic peptides. GBPs can also directly target bacteria that act on damaged vacuoles or escape into the cytosol, but the mechanism by which they recognize pathogens is unclear. The immune-related GTPase family (IRG) recognizes the components of the vacuolar structure of the pathogen. Host type I phosphatidylinositol 3-kinase (PI3K) produces 3,4,5-triphosphate phosphatidylinositol on mycobacterial phagosomes and binds to Irgm1 to form a soluble NSF attachment protein receptor (soluble NSF attachment protein receptor, SNARE) and autophagy-related proteins for fusion with lysosomes. From this point of view, both autologous and non-self-signaling require autophagy receptors and IFN-induced GTPases to eliminate intracellular pathogens as part of a cellular autonomous defense program.

Changing cellular components can affect the growth of microorganisms. One is to increase the concentration of substances in cells that are not conducive to the growth of pathogens. For example, in cells containing virus, the concentration of deoxynucleotide triphosphates (NTP) in the cytoplasm is significantly higher than that of cells without viruses. In order to eliminate excess dNTPs, human dendritic cells (DCs) and macrophages express interferon-induced SAMHD1, which hydrolyzes dNTPs to block the reverse transcription of human immunodeficiency virus (HIV)-1. cDNA synthesis. Another way to limit microbial growth is to consume amino acids. For example, IFN induces the degradation of L-tryptophan by indoleamine 2,3-dioxygenase (IDO), which is effective in inhibiting pathogenic microorganisms. In cells, phagocytic lysosomes and autophagosomes are the areas most susceptible to controlling inclusions, and acidified organelles kill and degrade internalized pathogens. The corresponding sterilization capacity comes from the synergy of several factors. Mammalian cells express a proton-dependent efflux pump, such as natural resistance-associated macrophage protein-1 (NRAMP1), which outputs Mn^{2+} and Fe^{2+} from vacuoles to prevent captured microorganisms obtaining these essential metals. The antimicrobial peptide destroys the outer membrane of the pathogen and further degrades the substance by a luminal protease, lipase, and glycosidase transported by the Golgi late endosomal pathway. Active oxygen and nitrogen oxidize and nitrosate pathogen lipids, DNA and proteins, respectively. Together they create an environment that is detrimental to most pathogens. The ATPase transported by the proton pump produces a low pH (~4.5–5.0) environment in these chambers and is maintained by a reverse transporter such as sodium-hydrogen antiporter-1 (NHE1), a low pH. The environment is optimal for maintaining lysosomal hydrolase activity and conversion of superoxide (O_2^-) to H_2O_2; the nitrogen-containing end product is the toxic free radical nitric oxide (NO). The transport of copper ions by the P-type ATPase Cu^{2+} pump (ATP7A) promotes the formation of toxic hydroxyl

radicals (·OH). Therefore, the concentration of various antibacterial activities in a professional organelle can promote microbial killing (Randow et al. 2013).

Autophagy can also play a role in microbial infection through innate and adaptive immunity. Autophagy is an important component of the innate immune response, and the innate immunity of cells against early stages of invading bacteria is through phagocytosis of bacteria and subsequent autophagy. For example, lipopolysaccharide (LPS) can induce autophagy through Toll-like receptor (TLR) 4, RNA interference inhibits TLR4 expression or inhibits TLR-related interferon response factor (IRF). Both expression of p38 and the like can block LPS-induced autophagy. Autophagy can also be involved in the regulation of adaptive immune responses in bacteria. In an adaptive immune response, cytokine secretion is different, and the interaction between autophagy and bacteria also changes. For example, cytokine IFN-γ secreted by CD^{4+} Th1 cells activates macrophages and induces autophagy levels of macrophages. Schmid et al. observed that in major antigen-presenting cells, such as DC and B lymphocytes, major histocompatibility complex (MHC) class II molecules are structurally very similar to the autophagy protein Atg8/LC3 (about 50–80%). RNA interference reduces the expression of autophagy proteins associated with autophagosome formation, leading to decreased antigen presentation capacity of MHC class II molecules and impaired adaptive immunity, suggesting that autophagy presents microbial antigens to MHC class II molecules. The process plays an important role.

The role and mechanism of autophagy in some pathogen infections closely related to human diseases will be introduced in the following.

29.2 Autophagy and Bacterial Enteritis

Salmonella is the main pathogen causing acute enteritis, among which *S. typhimurium*, *Salmonella enteritidis*, *Salmonella choleraesuis*, *S. typhimurium*, and *S. typhimurium* are more common. *Salmonella* infects epithelial cells and colonizes *Salmonella*-containing vacuoles (SCVs) that promote bacterial survival and replication. However, due to damage to the vacuole by the type III secretion system (T3SS) effector, most (about 15–20%) cytoplasmic bacteria or damaged vacuoles containing bacteria are rapidly enveloped by ubiquitin proteins and combined with autophagy receptors or LC3 positive neoautophagosomes. At least three autophagy receptors, p62, NDP52, and OPTN, recognize ubiquitinated *Salmonella* and target them to autophagosomes. The three receptors are independent and independent of each other during the recruitment of ubiquitinated *Salmonella*, and any depletion of autophagy receptors can lead to excessive proliferation of *Salmonella*. Autophagy receptors may exhibit different affinities for different polyubiquitin chains, and different receptors may recruit different effector proteins. There are at least two different types of polyubiquitin chain modifications in *Saccharomyces cerevisiae*: linear and K63, and post-translational modifications near the ubiquitin-binding domain also influence the selection of autophagy receptors. P62 will prefer K63 instead of K48,

and also binds to linear ubiquitin chains; OPTN combines with K63 and linear ubiquitin chains (van Wijk et al. 2012). NDP52 is recruited to bind to LC3 and TANK-binding kinase 1, TBK1, especially LC3C. TBK1 not only promotes antibacterial autophagy by phosphorylating OPTN and p62, but also regulates autophagosome maturation. At the same time, NDP52 is the only one of the three receptors that recognizes galectin-8 and binds to polysaccharides. Damaged SCV in the cytoplasm is more likely to induce autophagy than free *Salmonella* lectin-8, which interacts with host glycans exposed on damaged SCV, detects endosomal and lysosomal integrity, and senses bacterial infection. This direct binding allows NDP52 to be recruited through a non-ubiquitin-dependent pathway, but this effect is transient and subsequent recruitment of NDP52 via the ubiquitin-dependent pathway is still required (Thurston et al. 2012). In summary, galectin-8 is an early risk signal for host cells to detect intracellular bacteria.

Another ubiquitin-independent pathway that plays a role in autophagy-clearing bacteria requires a second messenger diacylglycerol (DAG), which is required for efficient autophagy of *Salmonella*. Although it is unclear how this process is initiated, SCV membrane damage may promote the production of DAG, which in turn raises protein kinase C (PKC) δ through c-Jun N-terminal kinase (c-Jun N-terminal) Kinase, JNK) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase pathway cause autophagy (Shahnazari et al. 2010). DAG and the ubiquitin-dependent pathway may play a role in targeting bacteria to autophagy, since bacterial autophagosomes can co-localize with DAG or ubiquitin.

Salmonella does not only passively evade host autophagy during infection, but has evolved multiple mechanisms to inhibit autophagy. For example, when *Salmonella* infects mouse macrophages, it may inhibit the signal transduction of TLR4 by activating AC6, thereby downregulating TLR4-mediated autophagy induction, which is beneficial to the survival of bacteria. *Salmonella* also promotes apoptosis of macrophages by causing the formation of small body-like structures of autophagy, a process that relies on T3SS. *Salmonella* T3SS secretes SipB protein, a protein with membrane fusion that, upon entry into host cells via T3SS, induces host cells to form a unique multilayer "onion membrane" structure containing mitochondria and endoplasmic reticulum can recruit autophagy proteins, resulting in a lack of energy supply to non-apoptotic death, indicating that *Salmonella* can induce and utilize autophagy through its own secreted proteins, aggravating cell damage. In addition, when *Salmonella* infects macrophages, SipB can be found in the swollen mitochondria, and the aggregation of autophagic vesicles by *Salmonella*-infected macrophages suggests that *Salmonella* can also cause autophagy by dividing mitochondria by SipB, thereby causing cell death (Fig. 29.1).

In addition, *S. typhimurium* that containing virulence plasmid genes (*Salmonella* plasmid virulence, spv) can suppress autophagy and reduce the ability of autophagic clearance of bacteria, beneficial bacteria survive in a host cell, resulting in serious damage to the cells. The autophagy inhibition of spv mainly depends on the virulence fragment spvB on its sequence. *Salmonella* containing spvB can inhibit autophagy in a variety of ways, such as promoting the expression of phospholipase D1 (PLD1) and

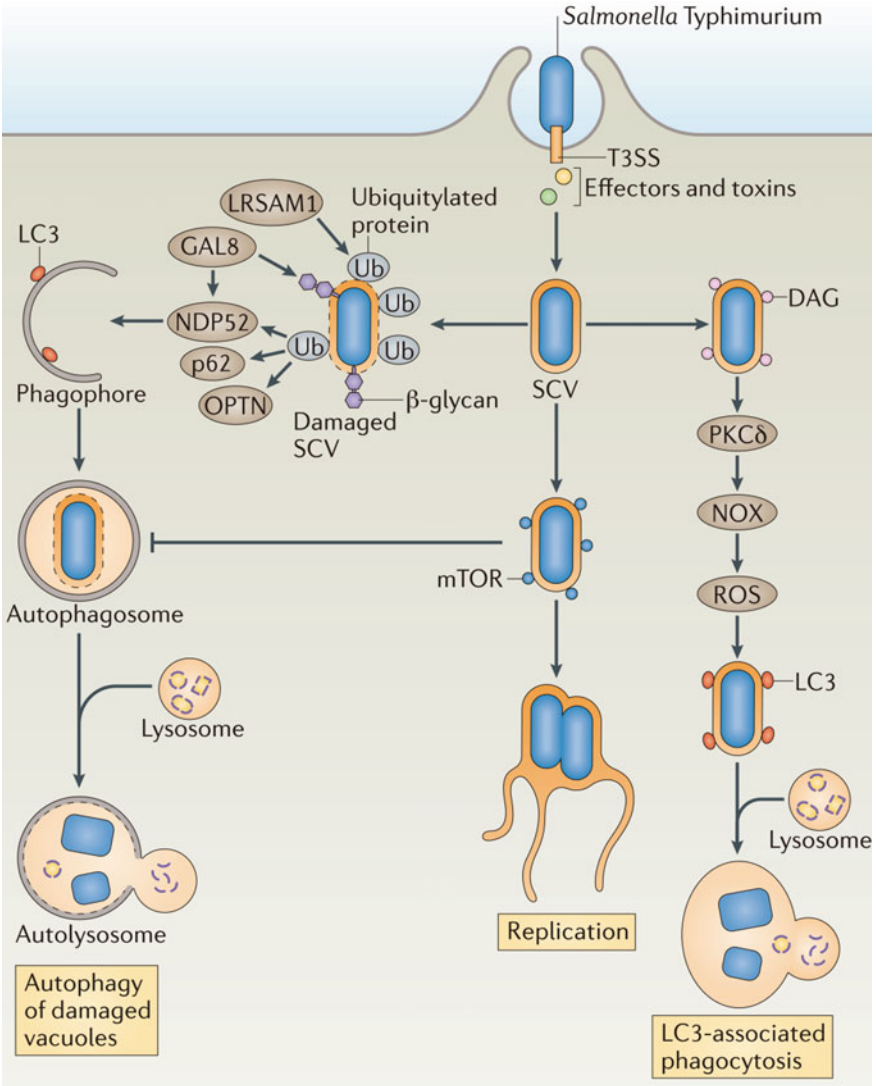

Fig. 29.1 The mechanism of *Salmonella* infection-induced autophagy. Ju Huang and John H. Brumell. Nature Reviews Microbiology, January 2014

enhancing the activity of PLD1, reducing the expression of Th1 type cytokine IFN-γ, and promoting the Th2 type cytokine interleukin (Interleukin, IL) -4 and IL-13 secretion. This inhibition of spvB can be reversed by rapamycin, because rapamycin is involved in PLD1 activity and Th1/Th2 shift, so spvB may activate mTOR signaling pathway mainly through AKT-dependent manner, affecting PLD1 activity and Th1/Th2 shifts to suppress autophagy. The Th1 cytokine INF-γ protects against

intracellular pathogen infection by promoting autophagy clearance of intracellular bacteria, and simultaneously interacts with the TLR4/MyD88 pathway, suggesting that the TLR4/MyD88 signaling pathway and the Th1-type response have synergistic effects in the body against pathogen infection.

Shigella is another common pathogen that causes intestinal infections. *Shigella flexneri* (*Shigella*) escapes into the cytoplasm, which replicates in the cytoplasm and spreads to other cells via actin polymerization. A portion of the bacteria is endowed with various mechanisms to survive in the cytosol and avoid autophagy degradation. After entering non-phagocytic cells, *Shigella* quickly escapes into the cytoplasm and replicates. Therefore, in order to successfully invade host cells, *Shigella* evolved to produce a mechanism to escape autophagy. Initially, the autophagy core protein ATG16L1 was rapidly recruited by NOD1 and NOD2 to the site where bacteria entered the plasma membrane. Once in the cytoplasm, *Shigella* uses the virulence protein IcsA to promote actin movement. The autophagy core protein ATG5 binds to IcsA and is capable of sequestering *Shigella* and degrading it by autophagy. However, the T3SS of *Shigella* also secretes IcsB (a protein encoded by the *Shigella* plasmid, localized on the surface of bacteria) which competes with ATG5 for binding to IcsA. Once bound to IcsA, IcsB protects *Shigella* from degradation, prevents ATG5 from recognizing IcsA, thereby blocking autophagosome-encapsulated *Shigella*, protecting bacteria from autophagy, and causing intracellular infection of *Shigella*. At the same time, septin also has a role in limiting the function of *Shigella*, and the prion-encapsulated *Shigella* co-localizes with p62, NDP52, and LC3 (Mostowy et al. 2010). Although most *Shigella* successfully escapes from autophagy, the vacuolar membrane residue can be recognized by galectin-8, polyubiquitinated and enters through interaction with the receptor protein p62. Degradation of membrane residues appears to be associated with control of downstream inflammatory responses that would otherwise be detrimental to host cells. In addition, Shiga Toxin (Stx) produced by *Shigella* can cause different mutations in toxin-sensitive cells such as THP-1, HK-2 cells, and tolerant cells such as human monocyte-derived macrophages. Typical autophagosomes were observed under electron microscope after Stx infection, but free Beclin1 and Atg5 proteins were present only in toxin-sensitive cells, but not toxin-tolerant cells, indicating autophagy induced by *Shigella* infection. The response is closely related to the type of host cell (Gomes and Dikic 2014).

29.3 Autophagy and Tuberculosis

Mycobacterium tuberculosis (*M. tuberculosis*) is a pathogen causing tuberculosis. It is a facultative intracellular parasite that mainly invades macrophages after infecting the body. *M. tuberculosis* can survive in vivo by preventing fusion of macrophages with endosomes and lysosomes until IFN-γ activates macrophages to transport tubercle bacilli to lysosomes. In 2004, Gutierrez et al. first reported that when rapamycin and IFN-γ were used to upregulate the autophagy level of host cells under starvation, *M. tuberculosis* was transported to the lysosomes of infected cells for degradation.

Papamycin can promote the attachment of phagosomes containing *M. tuberculosis* to the autophagy-related gene product Beclin1, which is beneficial to the generation and aggregation of phagosomes and the maturation of phagosomes. The results of transmission electron microscopy further confirmed that the bactericidal effect was mainly produced by promoting the fusion of phagosomes and lysosomes containing *M. tuberculosis*.

The transcriptional upregulation of autophagy by *M. tuberculosis* is a complex phenomenon involving multiple genes and multiple links of autophagy, including phagocytic vacuoles, autophagosomes, and autophagic lysosome formation, and substrate targeting/degradation. A variety of exogenous stimuli that cause autophagy can target *M. tuberculosis*, promote phagosome maturation, and thereby inhibit intracellular bacterial replication. To date, stimuli such as IFN-γ, IL-1β, TLR ligands, and vitamin D3 have been shown to target *M. tuberculosis* and promote phagosome acidification to activate autophagy. There is evidence that vitamin D metabolism plays an important role in the response of human macrophage hosts to mycobacterial infections. In vitro experiments have shown that vitamin D supplementation can improve the ability of macrophage to control *M. tuberculosis* infection in diabetic patients with low vitamin D levels. Vitamin D may inhibit the accumulation of lipid droplets induced by *M. tuberculosis* by inhibiting peroxisome proliferators-activated receptors (PPAR) γ, thereby affecting *M. tuberculosis* infection (Salamon et al. 2014). The transcription factor EB (TFEB) is an important regulator of autophagy activation, assembly, and targeted degradation. Recently, Kim et al. found that the nuclear receptor PPARα can play a role in *M. tuberculosis* infection by upregulating TFEB. The lack of PPARα increases bacterial load and enhances the inflammatory response to mycobacterial infections, which are achieved by upregulating Lamp2, Rab7, and Tfeb, which are involved in autophagy and lysosomal biosynthesis (Kim et al. 2017). This suggests that further research is needed to explore the possibility of using PPARα agonists in anti-tuberculosis treatment.

In addition to the *M. tuberculosis*, which relies on the autophagic lysosomal pathway to directly eliminate intracellular infections, ubiquitin-mediated activation of *M. tuberculosis* autophagy also promotes an innate immune response to *M. tuberculosis* infection. Although the ubiquitination process of *M. tuberculosis* remains unclear, p62, NDP52, NBR1, and OPTN have been shown to be involved in ubiquitin-dependent autophagy clearance, which can be recruited into ubiquitin-associated bacteria and attached to LC3. Intracellular bacteria are targeted to autophagosomes and degraded. Parkin, an E3 ubiquitin ligase, targets *M. tuberculosis* and its related structures to autophagosomes and promotes autophagy-mediated host resistance to *M. tuberculosis* (Manzanillo et al. 2013). Ubiquilin-1 (UBQLN1) recruits ubiquitin, p62 and LC3 to vacuoles containing *M. tuberculosis*, impeding bacterial growth and replication. Smurf1 is also an E3 ubiquitin ligase that catalyzes the ubiquitination of substrates for subsequent degradation in the proteasome. Franco et al. found that macrophages lacking Smurf1 were unable to recruit proteins required for autophagy and lysosome formation to *M. tuberculosis*-associated structures, mice with *M. tuberculosis* lacking the Smurf1 gene were more loaded, and lung inflammation was more serious, the survival rate is also lower (Franco et al. 2017).

M. tuberculosis can also regulate or utilize the host's autophagy response. Certain mycobacteria, such as the *Mycobacterium avium* complex (MAC), have evolved mechanisms to evade apoptosis and autophagy, allowing bacteria to freely infect adjacent macrophages during transmission. In addition, it is known that the *M. tuberculosis* virulent strain (H37Rv) infects the body and tends to induce the body to form an immune response mainly composed of a Th2-type immune response, while suppressing the Th1 type immune response. H37Rv can strategically upregulate IL-6 production against innate immunity, and IL-6 selectively inhibits IFN-γ-induced autophagy by attenuating the ATG12-ATG5 complex. As the infection time of *M. tuberculosis* increases, the production of IL-6 increases, and the use of antibodies to neutralize IL-6 significantly enhances IFN-γ-mediated intracellular bacterial killing (Dutta et al. 2012). In addition, *M. tuberculosis* H37Rv has a unique gene called "enhanced intracellular survival" (Eis). EIS can regulate autophagy, inflammation, and death of macrophages, and H37Rv-infected macrophages with *M. tuberculosis* Eis deletion mutations significantly increase autophagic vacuoles and autophagosome formation. EIS upregulates IL-10 expression and increases Akt/mTOR/p70S6K pathway activity to inhibit autophagy in macrophages (Duan et al. 2016). *M. tuberculosis* also inhibits the Ras-associated protein Rab7a, which blocks the autophagosome maturation into autophagic lysosomes. *M. tuberculosis* is precisely the type of this immune response that regulates cells to facilitate their own survival.

At the same time, *M. tuberculosis* in macrophages can evade immune attack by modulating microRNAs (miRNAs). miRNAs are a class of non-coding small single-stranded RNA molecules (about 22 nucleotides in length) that play important roles in macrophage function. miRNAs regulate immune responses through TLR signaling, play a key role in autophagy, and regulate host immunity during *M. tuberculosis* infection. Recently, after Gu et al. proposed that *M. tuberculosis*-infected macrophages, miR-23a-5p expression was upregulated with infection time and dose, and inhibited autophagy activation by TLR2-mediated signaling, increasing *M. tuberculosis* survival rate (Gu et al. 2017). Another study showed that Bacillus Calmette (Guerin, BCG) infected macrophages increased miR-20a expression, and miR-20a inhibited autophagy by targeting ATG7 and ATG16L1 mRNA. miR-20a also reduces the level of LC3-II in macrophages and promotes BCG survival (Guo et al. 2016). At the same time, the study found that induction of miR-33 in *M. tuberculosis* inhibits autophagy, lysosomal function, and fatty acid oxidation, allowing bacteria to survive and replicate (Ouimet et al. 2016).

There are many ways to stimulate autophagy and reduce the survival of mycobacteria in cells. miR-155 binds to the brain's enriched RAS homolog (Rheb), a negative regulator of autophagy, and promotes phagosome digestion. Selective serotonin reuptake inhibitor fluoxetine and epidermal growth factor receptor (EGFR) activity inhibitor gefitinib can also reduce *M. tuberculosis* in macrophages by promoting autophagy survival rate. The primary role of gefitinib is to block the activation of the EGFR-mediated autophagy inhibitory pathway p38-MAPK (Stanley et al. 2014). There is little in vivo study of ATG in *M. tuberculosis* infection, and ATG5 may play a role in early regulation of tuberculosis pathology and innate immunity of *M. tuberculosis* replication.

29.4 Infection of Other Intracellular Bacteria

When *Staphylococcus aureus* is infected, the autophagy transcriptional regulator HLH-30 (TFEB in mammals) can protect against bacterial load, but its mechanism of action remains unclear. Studies have shown that autophagy protects cells that are susceptible to pathogen-specific virulence mechanisms, rather than directly controlling cytokine expression. Both in vitro and in vivo experiments indicate that α-toxin is more likely to promote cell death in endothelial cells or epithelial cells of Atg16L1-deficient Atg16L1HM mice. Thus, although autophagy in other cell types is equally important during *S. aureus* infection, cells that maintain barrier function appear to be particularly susceptible to Atg16L1 deficiency in the presence of strains producing α-toxin (Maurer et al. 2015).

Porphyromonas gingivalis is an important pathogen of periodontal disease. It can be transported from early autophagosomes to late autophagosomes after being taken up by human coronary endothelial cells and human aortic endothelial cells. Autophagosome-lysosomal fusion or alteration of normal autophagy transport prevents autophagic lysosome formation, thereby colonizing in autophagosomes. LPS from *P. gingivalis* has a significant effect on autophagy of human gingival fibroblasts by inhibiting the PI3K/Akt/mTOR signaling (Liu et al. 2018).

In addition, autophagy plays an important role in many other bacterial infections. The role of autophagy in protecting mammalian cells from a variety of bacterial infections has been confirmed, but the response of autophagy to bacterial toxins is not fully understood. *Vibrio cholerae* secretes cytotoxic proteins such as *V. cholerae* cytolysin (VCC), which can cause vacuolation, cell lysis, and necrosis. The study found that VCC-induced vacuoles and LC3 co-localize. In mouse embryonic fibroblasts that knocked out the autophagy gene Atg5, VCC failed to induce vacuolization, and the ability of cells to survive against these toxins was greatly reduced, indicating that epithelial cells can resist this by induction of autophagy. In addition, autophagy can also synergize with cytoskeletal protein cells to inhibit pathogen infection. For example, autophagy-assisted spacer proteins limit pathogens to "protein-like compartments" to limit the spread of infection, but the specific process needs further study.

Autophagy, as an important part of the innate immune response and adaptive immune response, has expanded from maintaining the homeostasis of the internal environment to preventing the infection of intracellular microorganisms. Therefore, in-depth study of the mechanism of autophagy in bacteria and its interaction with cells, exploring effective and specific autophagy regulatory molecules remains the focus of current research. It is found that more virulence proteins or related molecules that destroy pathogens or use autophagy have positive clinical effects on the use of autophagy, which is a "double-edged sword", and to prevent and treat pathogen infection by regulating autophagy. In-depth study of the role of autophagy in intracellular pathogen infection has important guiding significance for discovering new

pathogenic mechanisms of bacteria, exploring new strategies for preventing and controlling infectious diseases by regulating autophagy pathways, and developing new drugs and improving vaccines.

29.5 Conclusion

Autophagy, as an important part of the innate immune response and adaptive immune response, has expanded from maintaining the homeostasis of the internal environment to preventing the infection of intracellular microorganisms. Therefore, in-depth study of the mechanism of autophagy in bacteria and its interaction with cells, exploring effective and specific autophagy regulatory molecules remains the focus of current research. It is found that more virulence proteins or related molecules that destroy pathogens or use autophagy have positive clinical effects on the use of autophagy, which is a "double-edged sword", and to prevent and treat pathogen infection by regulating autophagy. In-depth study of the role of autophagy in intracellular pathogen infection has important guiding significance for discovering the new pathogenic mechanism of bacteria, exploring new strategies for preventing and controlling infectious diseases by regulating autophagy pathways, and developing new drugs and improving vaccines.

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Chapter 30
Autophagy and Viral Infection

Yichuan Xiao and Wei Cai

Abstract Autophagy plays an important role in the fight against viral infection, which can directly remove the virus, interact with the viral protein, and at the same time regulate the innate and adaptive immunity and promote virus clearance. The virus has also evolved autophagy, which evades, antagonizes and utilizes autophagy, and regulates autophagy pathways, affects autophagy maturation, changes autophagy small body environment or changes the body's immune response type to promote or inhibit autophagy. This chapter introduces the possible mechanisms of autophagy during pathogen infection such as human immunodeficiency virus and hepatitis virus, in order to provide new methods for the prevention and treatment of viral infection.

Keywords Autophagy · Viral infection · Immune · Interaction

Autophagy plays a role in almost all aspects of virus recognition, infection, cell entry, replication of genetic material, and exocytosis. It also participates in interactions with the body and immune escape. Autophagy can transport viral proteins, nucleic acids, and intact viral particles into endosomes or lysosomal vesicles, further activating innate and adaptive immunity. Available evidence suggests that autophagy can play a protective role in different classes of viral infections. The multiple functions of autophagy in antiviral infections and their mechanisms of antiviral invasion and destruction demonstrate that autophagy plays an indispensable role in the struggle between host and virus. Similar to bacteria, the virus also formed a variety of self-protection methods to escape, antagonize, and even use autophagy in the long process of evolution. Therefore, autophagy plays an important role in a variety of viral infectious diseases such as AIDS, influenza, and viral hepatitis.

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30.1 HIV Infection

HIV is a retrovirus that mainly infects $CD4^+$ T cells, DCs, and macrophages. After infection, it can be divided into acute phase, asymptomatic phase, episode, and AIDS phase. HIV acts primarily on CD4+ T cells, which actively replicate to escape innate immune responses and severely deplete $CD4^+$ T cells, including infections, abortion infections, and bystander lymphocytes. This results in severe damage to the host's immune defenses and ultimately death due to opportunistic infections and cancer.

Autophagy plays a complex role in different stages of HIV infection. Early HIV infection requires autophagosomes to provide membrane support for viral replication. Both RNA knockout and pharmacological methods demonstrated the effect of autophagy on provirus formation in T cells and macrophages. The interaction of early HIV GAG precursors with autophagy protein LC3 promotes proper processing of GAG, suggesting that autophagy bodies may provide membrane support for viral replication (Kyei et al. 2009). IRGM is activated in certain infections, which are required for stimulation of autophagy and HIV replication. During HIV infection, IRGM is activated by interaction with the accessory protein NEF, and activation of IRGM promotes assembly of the ULK1/BECLIN-1/ATG16 complex to activate autophagy (Chauhan et al. 2015b). TLR8 can also interact with HIV to induce autophagy, and whether it is activated by TRAF6 activation of IRGM remains to be studied. In addition, the HIV negative-stranded antisense protein (ASP) binds to and co-localizes with LC3, promoting HIV infection by stimulating autophagy.

Despite the upregulation of autophagy during HIV infection, HIV can directly or indirectly affect the antiviral and immunological properties of autophagy. The purpose of HIV inhibition of autophagy is to prevent chelation of HIV proteins and degradation of lysosomes in autophagosomes. NEF interacts with BECLIN-1 to block the nuclear translocation of the transcription factor TFEB in an mTOR-dependent manner and inhibit autophagy maturation at the transcriptional level (Shoji-Kawata et al. 2013). VIF is also required for inhibition of autophagy, and its C-terminus acts directly with LC3B to inhibit autophagy (Borel et al. 2015). The HIV restriction factor TRIM5α regulates autophagy by promoting the activation of ULK1 and Beclin-1 assembly complexes as selective receptors, recognizing viral particles and recognizing the HIV capsid protein p24 as an autophagy substrate (Mandell et al. 2014). In addition, intracellular histone deacetylase (HDAC)-6 acts directly on VIF to promote autophagy degradation (Valera et al. 2015). The autophagy receptor p62 also selectively degrades the HIV transactivator TAT to limit HIV infection (Sagnier et al. 2015).

Therefore, stimulation of autophagy has both positive and negative effects on HIV infection, which is related to the degree of induction of autophagy. Moderate autophagy is effective against infected viruses in cells and inhibits HIV replication once autophagy exceeds a certain threshold. Stimulation of TLR8 leads to inhibition of autophagy-dependent HIV infection by cathelicidin microbial peptides and vitamin D receptors. In patients with stable long-term disease after HIV infection, the

level of autophagy protein in peripheral blood mononuclear cells is higher than that in patients with disease progression.

Autophagy of $CD4^+$ T cells and macrophages changes during HIV infection. HIV has multiple mechanisms that induce cell death through bystander $CD4^+$ T cells. HIV envelope glycoproteins (Env) on the plasma membrane of infected T cells have been shown to bind to CD4 receptors, and stimulate autophagy of bystander T cells through p53, Bax pathway, and Caspase 3 activation, leading to cell apoptosis. Expression of autophagy inhibitory genes such as Beclin-1 and ATG7 will result in low activation of Caspase 3 and inhibition of apoptosis. At the same time, autophagy also helps to induce HIV-infected cell death. p53 can upregulate the autophagy-related gene DRAM1, causing permeabilization of the lysosomal membrane and finally releasing the proteolytic enzyme cathepsin D to facilitate cell death (Laforge et al. 2013). The role of autophagy in aborted infected cells remains unknown.

In contrast to T cells, macrophages are not depleted during HIV infection, so macrophages are considered to be reservoirs of viruses. HIV infection can both induce autophagy and prevent autophagy degradation to avoid viral particle clearance, so HIV cannot induce cell death in these cells. Autophagy regulates both HIV replication and macrophage regulation of adaptive immunity DCs which may promote HIV infection, and intracellular viruses are transferred to T cells via HIV-derived cell–cell synapses, 5–10% initial state viruses can be lurking in the DC. The HIV envelope protein in DC activates mTOR and S6K to inhibit autophagy, thereby promoting the accumulation of HIV virus particles in DC. Furthermore, inhibition of autophagy by LC3 or ATG5 silencing increases the transfer of HIV to $CD4^+$ T cells in DCs (Blanchet et al. 2010). To prevent antigen presentation, HIV also reduces the expression of cathepsins to inhibit lysosomal acidification, preventing viral particles from being eliminated, thereby preventing antigen processing and presentation to T cells (Harman et al. 2009). Plasma-like DCs produce type I interferons and cause autophagy, whether it is infectious or non-infectious HIV (Zhou et al. 2012).

The effects of autophagy during HIV infection are multiple and can vary between different cell types. In summary, there is available research evidence that stimulation of autophagy-mediated lysosomal degradation is beneficial for enhancing the immune response to HIV. Rapamycin is a selective mTOR-inducing inhibitor of autophagy and may have a positive effect in controlling HIV infection. However, since rapamycin also has immunosuppressive effects, its therapeutic potential in HIV patients requires further validation. It is currently believed that vitamin D is expected to be an alternative drug, which has been shown to induce autophagy to inhibit HIV replication in primary human macrophages, but excessive vitamin D may damage the immune system and further control HIV infection. It has also been found in in vitro studies that HDAC inhibitors and microtubule targeting agents are expected to be agents of autophagy. HDAC inhibitors promote autophagy-mediated degradation of HIV particles and reverse infection of $CD4^+$ T cells during HIV latency. Fluconazole increases microtubule acetylation and induces autophagy by activating JNK1 and releasing it from the Beclin-1 complex via the autophagy inhibitor Bcl-2. Induced autophagy, in turn, prevents HIV from being transferred from DC to T cells (Chauhan et al. 2015a). Although in vitro studies have demonstrated that several of

these autophagy enhancers inhibit HIV infection, the side effects of their continued induction of autophagy in vivo have not been evaluated. The molecular mechanisms that regulate selective autophagy, such as the HIV-binding protein TRIM5α, have value in the development of new drugs that specifically enhance anti-HIV responses.

30.2 Hepatitis B Virus Infection

Hepatitis B virus (HBV) is a hepadnavirus that consists of four open reading frames, pre-S/S, pre-C/C, P, and X./Mid/large HBS envelope protein (small/medium/large hepatitis B s antigen, S/M/L HBsAg), HBV X protein (HBx), HBV e antigen, and HBV core antigen.

Both in vitro and in vivo experiments have confirmed that HBV can induce autophagy, and early autophagy can enhance viral replication. There are multiple pathways for HBV-induced autophagy; studies have reported that HBsAg can induce autophagy in cells by inducing endoplasmic reticulum stress and UPR (Li et al. 2011). HBx enhances PI3K activity, stimulates MAPK, ROS/JNK, and Sirt1 pathways to enhance autophagy, and interacts with VATPase to acidify lysosomes and block autophagic lysosome formation (Xie et al. 2018). SHB interacts with LC3 to induce autophagy (Ueno and Komatsu 2017). In summary, HBV primarily uses autophagy to enhance DNA replication. At the same time, HBV can also inhibit the maturation of autophagy, so that the virus is not degraded by autophagy (Zhou et al. 2016). Previous studies also reported that autophagy induced by HBV can degrade HBV capsid protein and limit viral replication by enhancing autophagy degradation. More research is needed about the relationship between HBV and autophagy.

30.3 Hepatitis C Virus Infection

Hepatitis C virus (HCV) is a single-stranded RNA virus of the Flaviviridae family of Hepatitis virus. The HCV genome encodes to produce 10 mature viral proteins, including core proteins, envelope proteins E1 and E2, p7 ion channel proteins and non-structural proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B.

Hepatocytes are the primary target cells for HCV, and HCV infection and entry into hepatocytes depend on the interaction between envelope glycoproteins E1 and E2, and host cell membrane protein receptors. These receptors include the four transmembrane proteins CD81, scavenger receptor B (SCRAB), clathin 1, CLDN1 and the like. In addition, low-density lipoprotein receptors, sulfated heparin, and the like are also involved in the interaction between viral particles and cell membranes. CD81 is involved in clathrin-mediated viral endocytosis into cells, and SCARB and CLDN1 are also involved in this process, possibly facilitating endocytosis by forming complexes. The virus enters the cell and then shells, releasing the viral genome into the cytoplasm, initiating translation of viral proteins and replication of viral RNA.

EGFR, ephrin receptor A2 and transferrin receptor are also involved in the entry of hepatitis C virus into hepatocytes.

Autophagy caused by HCV infection can be observed in hepatocytes of patients with cell culture or chronic HCV infection. HCV can induce autophagy indirectly through endoplasmic reticulum stress and oxidative stress. HCV viral proteins accumulate and activate PERK in the endoplasmic reticulum, thereby activating transcription factor 6 (ATF6) and IRE1, and then activating autophagy via the downstream effector molecule unfolded protein response (UPR) (Hetz 2012). At the same time, HCV-induced endoplasmic reticulum stress, NADPH peroxidase 1 and 4 expression and effects on mitochondrial function can induce oxidative stress (Ivanov et al. 2013). Studies have shown that the use of antioxidants to reduce oxidative stress in HCV-infected cells can reduce HCV-induced autophagy. HCV can also be directly induced by autophagy by recruiting autophagy proteins. P7 ion channel protein can bind to Beclin-1 to induce autophagy through the PI3KC3 complex (Aweya et al. 2013); HCV NS3/4A binds to mitochondria and IRGM, and with various autophagy-related proteins such as ATG5 and ATG10 Interactions regulate autophagy (Gregoire et al. 2011); NS5B can be screened in yeast two-hybrid to interact with ATG5 and ATG12 (Guevin et al. 2010); NS4B induces LC3 lipidation and is associated with Rab5, Vps34, and Beclin-1 that constitutes a complex (Su et al. 2011). In summary, HCV can induce autophagy by direct or indirect mechanisms. In addition, studies have shown that HCV can transiently regulate autophagic flow to optimize viral replication.

Autophagy can promote HCV replication, but there is still controversy about how autophagy affects HCV replication. The HCV cycle can be divided into five main steps, including viral entry, protein translation, RNA replication, viral assembly, and release. Autophagy is required for protein translation during HCV infection, and HCV inhibits premature fusion of autophagosomes and lysosomes to better utilize the membrane assembly of autophagosomes for HCV RNA replication complexes. Autophagy also plays a role in HCV release. Inhibition of ATG7 and Beclin-1 expression or inhibition of autophagy using chemicals can reduce the release of HCV from cells. Therefore, autophagy may affect multiple periods of the HCV cycle.

Moreover, HCV-induced autophagy can also help the body's inhibition of viral growth by interfering with the body's immune response. Invasion of the virus usually leads to the production of IFN in the body to exert an antiviral effect, thereby inhibiting the replication and survival of the virus. Inhibition of hepatitis C virus-induced autophagy can significantly upregulate IFN-mediated responses. Similarly, gene silencing of specific Beclin or ATG7 inhibits the growth of hepatitis C virus and activates the expression of interferon and ISG genes. HCV-induced autophagy can also deplete TRAF6, thereby limiting the production of host infectious agents. TRAF6 depletion occurs in the late stage of autophagy and is associated with delayed autoplasia. P62 can chelate TRAF6 to autophagosomes to degrade autophagosomes, minimizing the host's innate immune response (Chan et al. 2016). It can be seen that autophagy can inhibit excessive interferon antiviral response and promote replication of viral RNA to protect HCV-infected cells from being beneficial to virus survival.

Although HCV can induce autophagy to inhibit the innate immune response of IFN, IFN can also inhibit HCV replication by autophagy. In transgenic mice that specifically express HCV NS3/4A protease in the liver, Desai et al. found that when these mice were challenged with VSV or the synthetic HCV genome, a strong IFN-mediated response was produced, similar to the control mice (Desai et al. 2011). IFN-β, but not IFN-α, stimulates autophagy degradation of HCV NS3/4A following loss of HCV NS3 in transgenic mice. Kim et al. also reported that IFN-β can induce SCOTIN-binding to HCV NS5A and promote its transport to autophagosomes for degradation to inhibit HCV replication (Kim et al. 2016). Researchers also found that binding of IFN-λ1-a type III IFN to IFNλ receptor 1 inhibits the expression of ATG5 and γ-aminobutyric acid receptor-associated proteins and inhibits autophagy and HCV replication.

30.4 Autophagy and Other Viral Infections

Epstein-Barr virus is a human herpesvirus that is associated with many malignant tumors in genetically susceptible and immunosuppressed populations. Autophagy has an enhanced role in the early stage of Epstein-Barr virus infection, and its effect decreases as the level of Epstein-Barr virus lytic protein increases. At the end of the infection, the virus inhibits autophagy. Bafilomycin A1 inhibits autophagy or interferes with Beclin1 and can increase Epstein-Barr virus replication (De Leo et al. 2015). Epstein-Barr virus can also use host autophagy to regulate the level of intracellular oncoprotein LMP1, thereby affecting host cell proliferation (Hurwitz et al. 2018).

Cytomegalovirus (CMV) infection induces autophagy early, and later inhibits autophagy. In human umbilical vein endothelial cells, CMV induces autophagy through the mTOR signaling pathway, which enhances cell invasiveness and expression of ICAM-1 and VCAM-1 (Zhao et al. 2018). Recent studies have shown that trehalose can increase Rab7 levels to enhance autophagy and degrade viruses by promoting acid vacuolation (Clark et al. 2018).

Measles virus (MeV) belongs to the genus Paramyxoviridae, a common childhood infectious disease characterized by high fever, respiratory infections, and typical maculopapular rash. Attenuated MeV triggers autophagy by CD46-GOPC (Golgi-associated PDZ and coiled-coil motif containing) axis acceleration, and autophagosome formation by BECN1-VPS34 complex. In the early stages of infection, MeV-C interacts with IRGM autophagy to activate autophagy and promotes efficient replication of the virus. At the same time, autophagy also acts against MeV replication, and strong autophagy can also sense the virus and activate the IFN-I pathway through the innate immune receptor. Autophagy can promote MHC class II molecules to present MeV epitopes and induce CD4+ T cell responses. In contrast to attenuated strains, toxic MeV does not bind to the CD46 receptor, does not cause autophagy early, and does not detect significant changes in autophagy state. The relationship between autophagy and MeV infection remains to be explored (Rozieres et al. 2017).

HSV-1 is a double-stranded DNA virus and is a member of the α herpesvirus family. Autophagy plays an important role in HSV congenital infection. The toxicity of HSV- is mainly determined by the ICP34.5 gene of infected cells. ICP34.5 has a domain that binds to the autophagy regulator Beclin1, which blocks the autophagy process by interacting with Beclin1. HSV lacking a structure that interacts with Beclin1 (34.5 Δ68-87) does not inhibit autophagy from infected cells. 34.5 Δ68-87 mutant HSV mice can also enhance the ability of DCs to present MHC class I antigens (Budida et al. 2017).

30.5 Conclusion

Autophagy pathways and autophagy-related proteins play a crucial role in viral infection. The effect of autophagy regulation on the virus in infected organisms is not one-way, and is closely related to the stage of viral infection, which may promote virus replication or mediate virus clearance. There are many ways to regulate autophagy, modulating autophagy pathway or autophagy maturation, altering the environment of autophagy small body or the body's immune response type can promote or inhibit autophagy. At the same time, the virus also develops a new mechanism of resistance and utilization of autophagy in the process of combating autophagy, which makes the role of autophagy in viral infection more complicated. Elucidating the molecular mechanism of autophagy in viral infection will provide new methods for the prevention and treatment of viral infection.

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Part VI
Autophagy and Endocrine Diseases

Endocrine system is an important part of the physiological regulation of organism, and the nervous system, immune system together with the interaction of neurotransmitters, hormones, and cytokines and their receptors to achieve their own and cross-regulation, the formation of multiple bidirectional communication of the complex neuroendocrine-immune network system, jointly maintain the homeostasis. The classic endocrine system is composed of the pituitary gland, thyroid gland, parathyroid gland, adrenal gland, gonad, and pancreatic islet, it secretes more than 10 kinds of hormones.

At present, the understanding of endocrine has been greatly deepened, and its denotation has been greatly expanded, including nervous system, cardiovascular system, lung, liver, kidney, spleen, gastrointestinal tract, skin, adipose tissue, immune cells, etc. In recent years, autophagy is closely related to hypothyroidism, thyroid tumor, obesity, and diabetes, as well as obesity-related reproductive dysfunction. Therefore, more and more people begin to pay attention to the research on autophagy and seek more reasonable treatment methods for endocrine diseases based on autophagy regulation. Currently, the role of autophagy in cancer is complex. Autophagy is considered to be a double-edged sword. Under certain stress conditions, the up-regulation of autophagy may lead to cell death. With the selective degradation of cell components, autophagy also provides a pathway for cell survival, maintaining the circulation of nutrients and the energy generation of all eukaryotes.

Chapter 31
Autophagy and Thyroid Disease

Tao Tao and Huanbai Xu

Abstract Autophagy is a dynamic process, regulated by a variety of factors, and may play different roles in different thyroid diseases or in different stages of the same thyroid disease. Autophagy can mediate inflammatory response and immunity, which is closely related to the pathogenesis of thyroid autoimmune diseases. Therefore, it is still necessary to further understand the relationship between autophagy and autoimmune thyroid disease and hypothyroidism. With more and more studies on the relationship between autophagy and thyroid cancer, the relationship between the two is becoming more and more complicated. From the perspective of current studies, it is still worth pondering whether inhibition or activation of autophagy can be a valuable targeted therapy for thyroid cancer, and further research and efforts are still needed.

Keywords Autophagy · Thyroid autoimmune diseases · Hypothyroidism · Thyroid cancer

The thyroid gland weighs 20–30 g and is the largest endocrine gland in the human body. It is located on both sides of the upper trachea, below the thyroid cartilage, and is divided into left and right lobes. It is connected by a narrow isthmus in the middle, forming an "H" shape. The thyroid gland secretes thyroid hormones, which regulate the metabolism of the body and affect the growth and development of the organism. Thyroid hormone affects almost all organs and tissues of the body, and has effects on growth, development, metabolism, reproduction, tissue differentiation, and other functions. In addition, in the interstitial tissue adjacent to or between the follicular epithelium, there are scattered parafollicular cells (C cells) secreting calcitonin, which mainly regulates bone metabolism.

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Each thyroid disease has a direct or indirect link to iodine. Iodine deficiency can cause non-toxic goiter, thyroid nodule, thyroid tumor; too much iodine can lead to thyroiditis, hyperthyroidism, and hypothyroidism, which are related to toxic thyroid nodules and non-toxic multiple thyroid nodules. Nowadays, due to the implementation of iodized salt to prevent and control the prevalence of iodine deficiency disorders (IDD) in many countries, the number of patients with IDD goiter has been greatly reduced, and the proportion of thyroid tumor in thyroid diseases has been increasing year by year, and thyroid cancer accounts for 90% of all endocrine cancers.

31.1 Autophagy and Autoimmune Thyroid Disease

Studies have confirmed that autophagy can mediate inflammatory responses and immunity, which is closely related to the pathogenesis of autoimmune diseases. Autoimmune thyroid diseases (AITDs) are a group of predominantly T cell-mediated organ-specific autoimmune diseases, including Graves disease, and Hashimoto thyroiditis (HT), Graves eye disease, etc. AITD is the main pathological changes of cytokines involved in the mechanism of immune disorders. Studies have shown that autophagy-related proteins are involved in autoimmune thyroid lesions. During the pathogenesis of AITD, studies have found that autophagy activation in thyroid tissues of AITD patients is indeed abnormal, and autophagy inhibition promotes the processing and secretion of pro-inflammatory cytokines, which may aggravate AITD. A recent study showed that the polymorphism of autophagy-related genes would affect the susceptibility of AITD. In addition, as part of the thyroid polyprotein complex, caveolin-1 deficiency was also found to inhibit the autophagy activity of thyroid follicular cells exposed to Th1 cytokines, which may be the pathogenesis of AITD.

Hashimoto's thyroiditis (HT) is characterized by different levels of T, B lymphocyte infiltration, follicle destruction in thyroid tissue, which is a chronic inflammatory reaction of autoimmune diseases. A variety of mechanisms is involved in the onset of HT; when anti-thyroglobulin (Tg) immune starts, specific T lymphocytes induced thyroid follicular cells expressed major histocompatibility complex (MHC) class abnormal antigen expression, and further enlarge the inflammatory reaction, activation of lymphocytes, plasma cells, and macrophages to immune injury of thyroid, thyroid-specific antibody-mediated immune imbalance, which caused the occurrence of HT. Cytokines play an important regulatory and mediating role in the immune mechanism of HT. Studies have shown that autophagy is involved in the biosynthesis and secretion of cytokines, regulates the inflammatory response signal and participates in the inflammatory response. By upregulating the expression of MHCII, autophagy activates antigen-presenting cells and related cytokines, thereby affecting the differentiation of T cells. In recent years, it has been gradually discovered that Thl7, regulatory T cells (Tregs) are a kind of CD4+ T cell subgroup with independent effects, which play a role in promoting the release of inflammatory factors by thyroid follicular epithelial cells in HT, thus causing the production of thyroid autoimmune antibodies. Studies have shown that autophagy affects the immune response of Thl7

cells. An experimental study of autoimmune myocarditis showed that autophagy can promote the secretion of IL-17 by regulating the expression of beclin-1 and p62 proteins, thereby causing differentiation of myocardial plasma cells. In addition, activation of Tregs induces autophagy regulation mechanism to remove unnecessary protein molecules in the body, thereby inhibiting adverse immune response. In knockout autophagy genes AT97 or AT9 mice model, the results show that Tregs dysfunction causes the body's autoimmune inflammatory reaction. The above studies showed that autophagy is widely involved in the body's autoimmune regulation and plays an important role in Thl7/Treg cell immune imbalance. It is speculated that abnormal autophagy may be widely involved in the autoimmune injury of thyroid.

Furthermore, iodine is an important factor in maintaining normal thyroid function, but abnormal iodine intake can damage the thyroid, which is closely related to the occurrence of autoimmune thyroid diseases. Studies have shown that excessive iodine can induce and aggravate the occurrence of autoimmune thyroiditis in mice. Studies have found that excessive iodine can inhibit the autophagy-related protein LC3II expression by upregulating mTOR, which is related to the occurrence of HT. Iodine uptake in thyroid cells is mediated by sodium/iodine co-transporter (NIS), and studies have confirmed that NIS is degraded through the autophagic lysosome pathway. Excessive iodine can cause abnormal autophagy in thyroid follicular epithelial cells.

In addition, inflammatory factors and reactive oxygen species (ROS), as an important pathogenic mechanism, are involved in the inflammatory process of HT.ROS which are produced by all cells in an organism and are capable of redox-dependent regulation of different cellular functions, including response to stressors, angiogenesis, and cell proliferation. Excessive ROS can lead to pathological damage of thyroid follicular cells (TFCs) and the occurrence of autoimmune thyroid diseases (such as HT and GD). The study also found that autophagy activation was defective not only in IL-23 stimulated TFCs, but also in thyroid tissues of HT patients. The expression level of IL-23 in TFCs of HT patients was increased, leading to autophagy inhibition and ROS accumulation. Studies have shown that PKA/AKT/mTOR NF-κB signal pathway activation leads to excessive thyroid tissues IL-23 induced inhibition of autophagy activity and excessive accumulation of ROS. At the same time, excessive ROS also promotes the production of inflammatory reactions and pro-inflammatory cytokines and IL-23. The result is a positive feedback loop that worsens the severity of the disease.

The above studies suggest that the underlying mechanism of autophagy involved in the pathogenesis of HT is relatively complex and has not been fully elucidated.

31.2 Autophagy and Hypothyroidism

Clinical hypothyroidism is one of the important causes of secondary hyperlipidemia. Subclinical hypothyroidism is also closely related to dyslipidemia. Thyroid hormone action in the liver, adipose and other tissues and organs, through multiple pathways involved in the synthesis, decomposition, and transport of cholesterol and triacylglycerol. Therefore, thyroid dysfunction is often associated with abnormal lipid metabolism, notably the hypothyroidism cause of adverse effects on lipid metabolism and the resulting pay more attention to atherosclerotic cardiovascular disease. Recent studies have shown that the incidence of nonalcoholic fatty liver disease has doubled in hypothyroidism, affecting about 15% of patients. In addition, T3 and some TH analogs improved NAFLD in rodents on a high-fat diet (HFD). At the genome level, many genes that alter NAFLD expression are regulated by TH, further supporting the idea that defects in the TH signaling pathway may promote hepatic osteoporosis and liver injury. Two recent studies have also shown that hypothyroidism occurs more frequently in both young and elderly NAFLD patients. T3 stimulates the conversion of triglycerides into free fatty acids by increasing the mRNA expression and activity of hepatic lipase and transports them to mitochondria, indicating that thyroid hormone can regulate fat homeostasis through autophagy. T3 has previously been shown to reduce hepatic osteoporosis in cell cultures and rodent models by stimulating fatty acid oxidation in the liver through lipophagy and T3 and TH analogs. With the increase of oxidative phosphorylation, the production of mitochondrial ROS increases, which can lead to mitochondrial damage and cell death.

31.3 Autophagy and Thyroid Neoplasms

Thyroid cancer is the most common endocrine malignancy. In 2018, there will be more than 50,000 new cases of thyroid cancer, and the number of cases is on the rise. The vast majority (95%>) of thyroid cancers are from follicular epithelial cells, while the rest are from parafollicular cells. Follicular cell type thyroid carcinoma is mainly divided into three categories: well-differentiated thyroid carcinoma (WDTC), poorly differentiated thyroid carcinoma (PDTC), and undifferentiated or anaplastic thyroid carcinoma (ATC). WDTC includes thyroid follicular carcinoma (FTC) and thyroid papillary carcinoma (PTC). In the main types of thyroid cancer, A TC is the most rare and aggressive. Due to its high proliferation index and aggressive behavior, ATC at 6 months of disease-specific mortality was 69.4, and 80.7% at 12 months. The results showed that the PDTC and ATC tumor cells could not uptake iodine due to the lack of the expression of sodium iodine co-transporter. In addition, ATC cells may secrete thyroglobulin, and thyroid-stimulating hormone (TSH) may be difficult to resist due to the lack of TSH receptors on plasma cell membranes. The combination of these characteristics significantly restrict ATC routine radioactive iodine treatment

curative effect, the poor prognosis of ATC is partly due to its inherent of radioactive iodine and resistance to conventional chemotherapy.

However, most thyroid cancers belong to highly differentiated histologic subtypes and generally have a good prognosis after surgery and radioiodine ablation. Most PTC patients had a good prognosis after surgery and radiotherapy, and 5% of patients showed resistance to radiotherapy and chemotherapy. The activation of PI3K/A KT/mTOR pathway has been shown with a wide variety of tumor types including ATC associated with tumor progression and survival in patients with lower. Therefore, mTOR inhibition in molecular therapy is the focus of many anticancer studies and clinical trials. mTOR is a highly conserved protein kinase, and RAS/MA PK/ERK and PI3K/A KT/mTOR signaling pathways will merge with it. Its activation has been found to promote cell cycle progression and inhibit apoptosis in various human cancers. mTOR can be found in two structurally and functionally distinct multiprotein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Rapamycin is a first-generation mTOR inhibitor. It is an allosteric inhibitor that blocks mTOR-dependent downstream signaling. mTORC1 is sensitive to rapamycin, while mTORC2 is relatively insensitive to rapamycin. mTOR kinase inhibitor CZ415 inhibits the growth of human papillary thyroid cancer cells, and CZ415 has more obvious double inhibitory effect on mTORC1/2.

The limitations of rapamycin treatment in a clinical environment led to the development of the second generation of mTOR inhibitors, called ATP competitive mTOR kinase inhibitors. These inhibitors target on the mTOR kinase domain ATP area, thus inhibiting its catalytic activity. Mechanism advantage of these drugs is that they can inhibit mTORC1 and mTORC2 complex kinase activity, and activate blocking PI3K/AKT signal feedback. This kind of inhibitor is one of the representatives of AZD2014, and is currently in clinical trials of some solid tumors. Although we recently found AZD2014 has overcome ATC chemical resistant potential, through the study of mTOR inhibitors inhibit ATC is still in its infancy.

In addition, autophagy plays an important role in the homeostasis of the intracellular environment, and the imbalance in this process is also manifested in cancer cells. Recent in vitro preclinical studies have shown that autophagy is involved in the cytotoxic effect of chemotherapy drugs on thyroid cancer cells. Many oncogenes and tumor suppressor genes also play a role in the regulation of autophagy in thyroid cancer, and some acquired regulatory factors involved in thyroid cancer also affect autophagy.

31.3.1 The Role of Autophagy in the Progression of Thyroid Cancer

Recently, autophagy has been shown to promote cell survival in response to nutrient deprivation and exposure to inflammatory or pro-apoptotic stimuli.

When the body is under pathological conditions, such as calcium overload, ischemia, and hypoxia, cells can initiate autophagy to remove damaged mitochondria, ensure the survival of normal cells, and decompose organelles to obtain supplementary energy. However, a large number of autophagy can still lead to excessive cell self-digestion, causing irreversible cell damage and cell death. It has been previously reported that the major signal transduction pathways connecting thyroid cancer with autophagy regulation are RAS-RAF-ERK and PI3K-AKT-mTOR pathways. So, inhibit RAS/MAPK/ERK or PI3K/AKT/mTOR pathway composition may be ATC valuable treatment. RAS-RAF-ERK pathway controls mTOR-dependent pathway by sensing amino acid deletion. Abnormal signals through RAS/RAF/MEK/ERK cascade are believed to be involved in the occurrence and development of thyroid cancer. For example, RET/PTC rearrangement activates Ras-Raf-MAPK cascade (Fig. 31.1).

However, the most common phenomenon in this signaling pathway of thyroid cancer is that thyroid cancer is related to the activation of oncogene BRAF, and BRAFV600E mutation leads to the activation of BRAF kinase. The study also found that BRAFV600E mutations in the tumor cells, NIS, TPO, lower TSHR gene expression. PTC often exists BRAF point mutations (30–69%) and RET cancer gene rearrangement (40–70%), and is also visible in the ATC and PDTC. The activation of

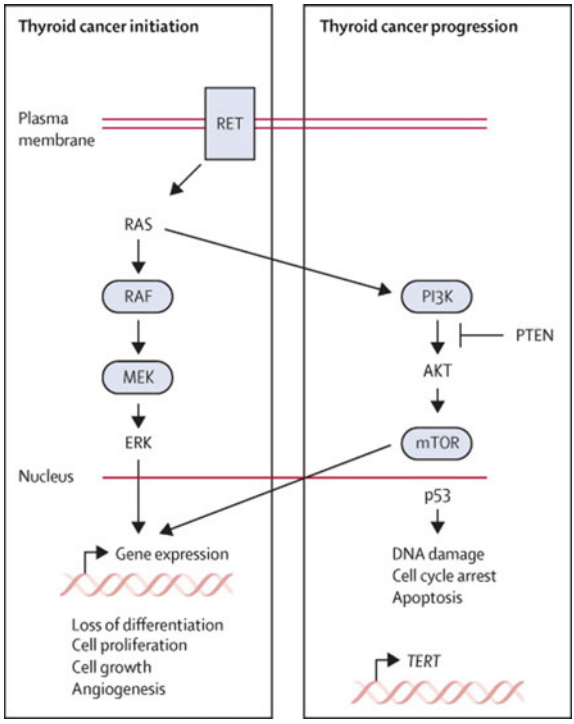

Fig. 31.1 The key molecular signaling pathways for thyroid cancer are shown here. The box on the left shows the mitogen-activated protein kinase pathway, which is activated by most thyroid cancer mutations. These events are thought to initiate the development of thyroid cancer and lead to changes in gene expression that promote cell proliferation, cell growth, angiogenesis, and loss of differentiation. The box on the right shows changes in pathways in advanced thyroid cancer that are believed to promote tumor progression. This includes changes in the PI3K mTOR pathway, p53 tumor suppressor, and TERT promoter

the RAS mutations is associated with the invasive and prognosis of thyroid carcinoma, and PTC rare RAS gene mutation (10–20%). The FTC is often RAS mutation (18–52%) and/or PPARγ/PAX8 restructuring (25–56%). RAS mutation may activate MAPK and PI3K/A KT signaling pathways. PTEN mutation may also exist in FTC, and found in the PTC and ATC PTEN silence may be due to the PI3K–AKT antiapoptotic pathways of constitutive activation. In addition, there may be a PI3K/AKT signaling pathways of genetic changes in ATC. Meanwhile, P53, a tumor suppressor gene associated with autophagy in thyroid cancer, may also be mutated. In the same way, often can be found NRAS and HRAS gene mutations in the FTC and ATC, activate ERK1/2-MEK1/2 and PI3K–AKT pathway, by raising the baseline level of autophagy to affect the metabolism of cancer cells. In addition, TGF- 1/Smad signaling has been confirmed to be important in thyroid cancer. Especially in ATC, it is more common to find that TGF, Smad, and AKT interactions.

In addition, recently found in PTC, a kind of tumor suppressor genes GAS8–AS1, can through regulating A TG5 PTC influencing the expression of tumor cell proliferation. Although its function mechanism has not yet been fully elucidated, research shows that GAS8-AS1 may act as a potential therapeutic target on PTC treatment; and methylation of DNA in epigenetic, histone acetylation and miRNAs were also involved in the autophagy process of thyroid carcinoma. For example, MirR221/222 and miR21 act on PTEN and maintain AKT excessive activation, and mTOR rely on indirect negative regulation of autophagy. In PTC and A TCS miR21 upregulate, at the same time in the PTC miR221/222 also upregulate. And MiR-144 can inhibit ATC cell autophagy by downregulating TGF-α, which enhanced sensitivity to cisplatin ATC cells.

31.3.2 Potential Application Value of Autophagy in the Treatment of Thyroid Cancer

Autophagy targeted therapy is currently considered to be a valuable and important strategy for tumor chemoradiotherapy resistance. A large amount of experimental data indicates that the efficacy of this therapy is strictly dependent on the actual level of autophagy in tumor cells, which depends on genetic mutations and epigenetic phenomena in addition to the dynamic effects of tumor microenvironment. With regards to the treatment of thyroid cancer, in addition to radiotherapy, new molecular therapies have emerged, such as protease inhibitors, small molecule tyrosine kinase inhibitors, angiogenesis inhibitors, and angioblockers, and other regulatory factors, which have also been proven to affect the autophagy process.

For example, Apatinib, an inhibitor of vascular endothelial growth factor receptor-2, has been shown to promote the anticancer effect of gastric cancer, lung cancer, breast cancer, and other malignant tumors. Previous studies have shown that apatinib accelerate the apoptosis of ATC cells, and confirmed that apatinib downregulates p-AKT and p-mTOR signals through the AKT/mTOR pathway, inducing autophagy

and apoptosis in human ATC cells. In addition, the combination of apatinib and the autophagy inhibitor chloroquine in vivo and in vitro can significantly inhibit tumor growth. These results suggest that both autophagy and AKT/mTOR signaling are involved in apatinib-induced ATC cell death. Patients with ATC may benefit from new anticancer drugs, while molecular-targeted therapies combined with autophagy inhibitors show promise for improved treatment.

Autophagy is widely believed to play a regulatory role in the proliferation and death of cancer cells. The dual role of autophagy in the development and progression of cancer has important clinical significance in the treatment. In addition, autophagy is a cellular pathway that promotes immune regulation and is involved in pathogen control and autoimmunity. Its regulation is multifactorial, including many epigenetic pathways that modify DNA binding histones to induce autophagy-related mRNA synthesis or microRNA and splicing related mRNA degradation, leading to autophagy inhibition. The recognition of epigenetic pathways involved in autophagy and autoimmunity may facilitate the use of a new class of epigenetic drugs in these important diseases. In fact, therapeutic drugs that promote and inhibit autophagy have shown efficacy in in vitro and in vivo tumor models, as well as in clinical trials. These contradictory results can be explained by different actual levels of autophagy in tumor cells, which may be due to different experimental models, different genetic and epigenetic backgrounds, and may be affected by matrix factors including angiogenesis, the presence or absence of cytokines, and the interaction between fibroblasts and macrophages.

In conclusion, the mechanism of the relationship between autophagy and thyroid cancer needs to be further studied to clarify the role of autophagy-related proteins in diagnosis and treatment, so as to find a better treatment.

31.4 Conclusion

Autophagy is a living phenomenon widely existing in eukaryotic cells, by which living organisms maintain the balance of protein metabolism and the stability of cell environment. Autophagy is a dynamic process, which is regulated by many factors. Autophagy may play different roles in different thyroid diseases or different stages of the same thyroid diseases. Therefore, it is necessary to further study the activity of autophagy in AITD and elaborate the relationship between autophagy and thyroid autoimmune diseases. The autophagy pathway in the process of thyroid cancer is regulated by oncogenes and tumor suppressor genes, so drugs that affect the autophagy pathway are very likely to become the targeted drugs for the treatment of thyroid cancer. In addition, epigenetic and miRNAs are also involved in the autophagy process of thyroid cancer. With more and more studies on the relationship between autophagy and thyroid cancer, the relationship between autophagy and thyroid cancer presents a complicated trend. From the current study, it is still worth pondering whether inhibition or activation of autophagy can be a valuable targeted therapy for thyroid cancer, and further research and efforts are still needed.

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Chapter 32
Autophagy and Obesity and Diabetes

Tao Tao and Huanbai Xu

Abstract The prevalence of obesity is increasing rapidly and is closely associated with a variety of metabolic diseases. Recent studies have suggested that autophagy is likely to play an important role in the development of obesity and may be related to insulin sensitivity. Autophagy may be involved in the browning of white adipose tissue and may also affect the metabolic balance of lipids. Autophagy can degrade cytoplasmic lipids by lipophagy in hepatocytes. Furthermore, Autophagy in hepatocytes helps prevent NAFLD. The study of autophagy in glucose metabolism is still in a very preliminary stage. Changes in autophagy activity play an important role in the development of insulin resistance in diabetes and many metabolic diseases. Therefore, it is still worth further exploration on the deeper mechanism of oxidative stress induction of insulin resistance to autophagy and whether there will be corresponding complications to the body.

Keywords Autophagy · Obesity · Diabetes · Lipophagy · Glucose metabolism

32.1 Autophagy and Obesity

32.1.1 Etiology and Epidemiology of Obesity

The decline of physical activity owing to changes in diets, transportation patterns, and more common sedentary work around the world, ultimately brings on energy imbalances. Obesity occurs consequently when the intake of energy exceeds consumption. Obesity is closely correlated to a number of health problems, including hypertension, type 2 diabetes, fatty liver, atherosclerosis and degenerative diseases

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(such as dementia), respiratory diseases, and a variety of cancers. According to the latest WHO statistics, the global prevalence of obesity has nearly tripled since 1975. More than 1.91 billion adults are overweight, of whom more than 650 million are obese. It is estimated that 41 million children under the age of five and more than 340 million children and adolescents aged 5–19 are overweight or obese (Gonzalez-Muniesa et al. 2017). There are 46 million obese adults in China, and the obesity population is getting even younger, and the obesity rate among young people continues to increase. Therefore, it is urgent to control obesity in China. The WHO has identified obesity as the world's largest chronic disease and one of the top medical problems of the twenty-first century. Therefore, the prevention and treatment of obesity will have a huge impact on the improvement of health and economic development of China and even the whole mankind. A better understanding of obesity would certainly help solve the predicament.

32.1.2 Relation Between Obesity and Autophagy Level

Obesity is a chronic disease resulting from the interaction of multiple factors including genetic and environmental factors, which affects the metabolism and physiology of multiple organs. Autophagy is an important physiological process that provides nutrients to maintain important cellular functions in the state of nutrient deficiency, while removing excessive or damaged organelles and misfolded proteins and lipids in the state of nutrient overabundance. Obesity is related to excessive nutrition and may inhibit autophagy, that is to say, the autophagy level in the body of obese people is likely to be inhibited, which will lead to the accumulation of harmful substances in the cells and cause mitochondrial oxidative stress and endoplasmic reticulum stress, resulting in insulin resistance (Ost et al. 2010). However, whether the changes in autophagy level during the whole development process of obesity is a dynamic process has not been confirmed. However, it is certain that restriction of energy intake increases autophagy activity in obese people, and the increase of autophagy level is related to insulin sensitivity and viability. Interestingly, studies have also shown that the autophagy level of adipose tissue in obese patients, especially in the greater omentum adipose tissue, is upregulated; the LC3 level of adipose cells in obese patients is increased, the number of autophagosomes is increased, and the mitochondrial content and the number of lipofuscin particles are decreased by electron microscopy. In addition, the extent of autophagy activation of omentum fat cells in obese patients was related to the extent of obesity, visceral fat distribution, and fat cell hypertrophy. The higher BMI, greater omentum visceral fat distribution, larger adipocyte diameter, and higher expression level of autophagy gene ATG5 mRNA were found in individuals (Kovsan et al. 2011). Therefore, it is urgent to further study the dynamic changes of autophagy level during the development of obesity in the future, so as to obtain and determine the true changes of autophagy during the development of obesity.

32.1.3 *Effect of Autophagy on Adipose Tissue and Consequent Obesity*

Adipose tissue is the core of obesity and metabolic diseases. Triglycerides are mainly stored in adipose tissue and metabolized in the liver. Abnormal lipid metabolism plays a crucial role in the occurrence of obesity. Autophagy is involved in the regulation of lipid metabolism. Studies have shown that there seems to be a mutual regulatory mechanism between insulin signal transduction and autophagy activity, suggesting the role of autophagy in insulin resistance. Upregulation of autophagy may promote the conversion of white fat to brown fat, thereby regulating energy consumption and obesity. Upregulation of autophagy in hepatocytes may increase lipid storage and affect triglyceride balance and fatty liver disease. Current evidence suggests that autophagy maintains lipid metabolic balance by regulating lipid storage in fat and liver tissues (Fig. 32.1).

The effects of autophagy on adipose tissue mainly include two aspects. On one hand, autophagy is necessary for cytoplasmic recombination and mitochondrial clearance during fat formation. On the other hand, autophagy may be involved in the regulation of mitochondrial metabolism in mature adipocytes. Thus, autophagy is necessary for adipocyte differentiation and lipid droplet accumulation. When the nutritional status changes, autophagy can increase or decrease the degradation of lipid droplets according to the needs of the body, thus playing an important role in regulating lipid metabolism in fat cells and maintaining the functions of fat cells. Studies have shown that knockout of Atg7 gene inhibits fat-specific autophagy and reduces total fat accumulation in adipose tissue (Frudd et al. 2018). Thus, it is speculated that the lack of autophagy in adipose tissue inhibits the formation of lipid droplets, thereby inhibiting fat accumulation. However, the accumulation of triglycerides in adipose tissue generally occurs when the nutrient supply is adequate, while autophagy is just at a low base level, i.e., autophagy at a low base level may be necessary for the accumulation of fat in adipose tissue. The generation of obesity

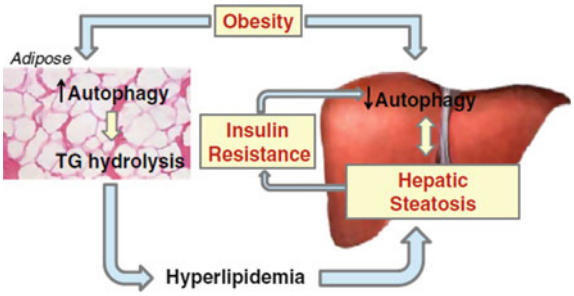

Fig. 32.1 Pattern of autophagy in liver and adipose tissue. The change of autophagy level in obese patients, which not only affects the storage and metabolism of triglycerides, but also is related to the development of hepatic steatosis and insulin resistance, and regulates the lipid balance through the function of fat tissue and liver—the two main lipid storage organs

is related to the excessive accumulation of lipids in white adipose tissue, and the oxidation and decomposition of lipids in brown adipose tissue generate a lot of heat energy. Brown fat was once thought to be extremely rare in adults, but it has recently been found to be present in significant amounts in adults. Regulating adipose differentiation to promote brown fat production is a new approach to obesity treatment. If autophagy is inhibited in white adipose tissue, normal differentiation is blocked, leading to brown adipose changes in white fat, also known as beige adipose tissue. In animal studies, browning of white fat resulted in weight loss and increased insulin sensitivity in mice. How autophagy regulates adipose differentiation is the key problem. Different in vivo and in vitro studies have proved that autophagy is beneficial to white fat differentiation, and autophagy promotes the degradation of a large number of cytoplasm, including mitochondria, which may be the key in this process. Electron microscopic analysis of differentiated preadipocytes showed that cytoplasmic components, including mitochondria, were immobilized in autophagy (Sarparanta et al. 2017), suggesting that autophagy was involved in cytoplasmic recombination observed during adipogenesis, and that fat production was significantly reduced in mice lacking autophagy.

Studies have shown that knockout of some important autophagy-related genes (ATG13, ULK2, RB1CC1, ATG5, ATG7, BECN1) can inhibit adipocyte differentiation (Frudd et al. 2018). The ULK1-ATG13-FIP200 complex-mediated mTOR signaling pathway induces autophagy. BECN1 is necessary for autophagy to influence the differentiation of 3T3-l1 adipocytes (Yujie et al. 2014). ATG7 gene knockout inhibits autophagy of 3T3-L1 preadipocytes, resulting in retardation of fat synthesis, decreased expression of adipocyte differentiation markers and decreased lipid storage. Similar results were obtained in white adipose tissue-specific ATG7 knockout mice (Zou et al. 2017). Compared with wild type mice fat cells, in white adipose tissue specificity ATG7 knockout mice fat cells contain smaller multicellular lipid drops; cytoplasmic volume increase, increases the number of mitochondria, and β-oxidation rate of free fatty acids and the expression of coupling protein 1 (UCP1) will increase, which shows that the ATG7 knockout increase white fat cells in number and function of mitochondria (Xu et al. 2018). In other words, the knockout of ATG7 endows white adipose tissue with the characteristics similar to brown adipose tissue. The results of these animal experiments suggest that autophagy plays an important role in lipogenesis. However, in the case of increased PGC1 level, the increase of mitochondrial content in ATG7 KO mice may also be caused by the increase of mitochondrial biosynthesis (Christoph and Spiegelman 2008). ULK1 form complexes with mTORC1, ATG13 to induce autophagy starting, but the specific role of ULK1 in adipocyte differentiation is unclear (Papinski and Kraft 2016). It is found that 3T3-L1 cells in the case of ULK1 lack also still could differentiate into fat cells (Sarparanta et al. 2017). Another factor that determines the accumulation of triglycerides is the degradation of lipid droplets by lipolysis. For more than 40 years, lipohydrolase has been regarded as the only enzyme needed for fat decomposition. However, other proteins such as MGL, ATGL, perilipin, and CGI-58 (ABHD5) have recently been found to be involved in fat breakdown. Perilipin phosphorylation promotes the action of HSL and the occurrence of lipolysis. Insulin

stimulates the insulin receptor of adipocytes and phosphodiesterase-3B to promote cAMP degradation and anti-lipolysis. The process of fat breakdown is more complex than previously thought. Singh et al. reported that autophagy is necessary for the process of fat decomposition, and autophagy is selectively involved in the process of lipid droplet transfer to lysosomes and degradation. They found that on an empty stomach, autophagy marker proteins, including LC3 and other proteins, enter the lipid droplets to form double-membrane vesicles or so-called autophagosomes. The autophagosomes containing lipids fuse with lysosomes to form autophagosomes, and the lipid is then degraded (Cuervo et al. 2009). Since adipose tissue plays a central role in the energy balance control of the whole body, and the mitochondrial function of adipose cells is also essential in lipid storage and hormone secretion, the regulation of autophagy of adipose tissue is bound to produce beneficial effects on the metabolism of the body.

32.1.4 Autophagy in the Liver

In general, autophagy is non-selective, but recent studies have found that autophagy can also specifically choose to degrade specific macromolecules or organelles, and can be divided into lipophagy, mitochondrial autophagy and peroxisomal autophagy according to the different substrates of autophagy, namely, selective autophagy (Arakawa et al. 2017). The key role of autophagy in hepatocytes is lipophagy, which is the process of selectively degrading cytoplasmic lipid droplets by the formation of autophagosomes, the degradation of some lipid droplets, and then the fusion with lysosomes. The physiological role of lipophagy in liver cells is to provide free fatty acids, which are then broken down by β-oxidation (Ward et al. 2016). Recent studies conducted have shown that lipophagy occurs in different types of cells including immune cells. The role of lipophagy does not seem to be limited to energy production. For example, fasting may induce lipophagy in the hypothalamus, increasing intracellular levels of free fatty acids that are used to produce agouti-related proteins (AgRP) that may increase appetite but reduce energy expenditure (Schulze et al. 2017b). The molecular mechanism of lipid droplet selective autophagy remains to be elucidated. Shibata et al. found that the formation of lipid droplets in hepatocytes increased when the hepatocytes were starved, and the formation of lipid droplets also decreased when the Atg7 gene of hepatocytes was knocked out to inhibit the level of autophagy. Meanwhile, the autophagy marker lc3-ii can be located on the surface of the isolation membrane and lipid droplets of the autophagosome. This study suggests that autophagy may play an important role in the formation of lipid droplets. However, this study is only concerned with lipid droplet formation during starvation, suggesting that lipid droplet formation may be necessary for lipid degradation, at least in liver cells. By inhibiting autophagy through the insulin and amino acid-mTOR signaling pathway, it can regulate short- and long-term lipid metabolism (Angelini et al. 2016). Short-term inhibitory effects are mainly produced by the

mTOR complex, while long-term regulatory effects are mainly mediated by insulin-induced Akt/PKB and mTOR inhibiting transcription factors FOXO and TFEB regulating autophagy gene transcription. When the mice were fed a high-fat diet, this autophagy-mediated fat breakdown in their livers was impaired, creating a vicious cycle: the more fat accumulated, the less fat was removed by autophagy-mediated fat breakdown. They also found that autophagy declines with age, which could explain why fat accumulation becomes easier with age. More importantly, they found that in cultured hepatocytes or mouse livers, Atg7 knockout inhibited autophagy, leading to increased hepatocyte and liver fat accumulation. Therefore, autophagy is a necessary process for stored lipolysis or lipolysis of liver cells. In the state of obesity, the level of autophagy in liver cells decreased significantly. It may be related to the following reasons: (1) The increase of obesity-induced calpain, Calpain II can degrade ATG7 and lead to autophagy defects, and the rapid inhibition of Calpain II can restore the expression of ATG7. (2) In obese mice with hepatic steatosis, autophagy inhibitor mTOR was detected to be over-activated in the liver, which may be related to the increase of amino acid concentration caused by excessive nutrition. Previous studies suggest that infusion of mixed amino acids can over-activate the S6 kinase downstream of mTOR, inhibit the phosphorylation of IRIS-1, and eventually lead to the development of insulin resistance in liver and skeletal muscle. (3) Akt/PKB, a key molecule of the insulin signaling pathway, reduced the level of autophagy in the liver of obese mice, but no upregulation of autophagy was found in obese mice whose secretion of insulin by cells was destroyed by streptozotocin, while the opposite was found in non-obese mice. The cause of these differences is still unclear, but shows that the high insulin hematic disease may also help obese mice autophagy. (4) It has been reported that autophagosome substrates are impaired in obese ob/ob mice, mainly including defects in lysosomal acidification and reduced cathepsin L, accompanied by the number of autophagosomes and the normal fusion of autophagosomes with lysosome increase. Clinical studies have found that the expression of hepatic cathepsin B, D, and L in NAFLD patients is significantly reduced. (5) There was a defect in the fusion of autophagosomes and lysosomes in the liver of obese mice induced by high-fat feeding, which was caused by the change of membrane lipid structure caused by high-fat feeding. Autophagy defects in hepatocytes are associated with decreased lysosomal degradation rate, leading to further aggravation of endoplasmic reticulum stress. Excessive nutrition can also induce the increase of endoplasmic reticulum stress in inflammatory environment. The decrease of hepatic autophagy and the increase of endoplasmic reticulum stress further aggravate insulin resistance. However, in the process of disease, autophagy degradation is a dynamic process, and the study on the change of autophagy flux is still a great challenge in this field. Lipophagy in hepatocytes is speculated to prevent NAFLD (Zhang et al. 2018). Studies have shown that lipid toxicity effects in NAFLD can inhibit autophagy (Schulze et al. 2017a). Enhancement of autophagy reduces hepatic fat deposition. The inhibition of autophagy in NAFLD further leads to the excessive activation of the SQSTM1-Keap1-NRF2 pathway (Ueno and Komatsu 2017).

32.2 Conclusion

In conclusion, autophagy does change in some way in obese people, and this change is likely to play an important role in the development of obesity. Activation of autophagy may have been involved in the development of insulin resistance before obesity-related metabolic diseases. Autophagy regulates the lipid balance of obese people through the role of adipose tissue and liver, the two major lipid storage organs. It not only affects the storage and metabolism of triglycerides, but also affects lipid metabolism through the assembly of lipoprotein. More evidence suggests that autophagy plays an important role in the degradation of apolipoprotein B (the main structural protein of the very low-density lipoprotein). Therefore, autophagy plays a very complex role in lipid balance, affecting the storage of lipids in different tissues and promoting lipoprotein metabolism pathways, which need to be further studied. In addition, it remains to be further explored whether downregulation of autophagy can change the deeper mechanism of the normal process of adipose tissue differentiation and whether it will have adverse effects on the body.

32.3 Autophagy and Diabetes

Diabetes is an endocrine and metabolic disease characterized by chronic hyperglycemia and/or relative insulin deficiency. It is one of the most common chronic diseases. Clinically, there are mainly two types of insulin-dependent (IDDM, type 1) and non-insulin-dependent (NIDDM, type 2). Diabetes may also manifest during pregnancy and in other cases including drug or chemical toxicity, genetic disorders, endocrine disorders, insulin-receptor disorders, and pancreatic exocrine disorders. In addition, the incidence of type 1 diabetes is increasing year by year in western countries. The increase in the incidence of type 2 diabetes, especially in developing countries, is accompanied by changes in urbanization and lifestyle, indicating that environmental factors are an important contributor to diabetes.

International Diabetes Federation (IDF) released the latest global diabetes prevalence; as you can see, the global number of diabetes patients has reached 370 million in 2011, 80% of them in developing countries, which estimates that by 2030 the world will have nearly 550 million diabetic patients, and the number of diabetes has become the world's first in China. According to a recent epidemiological investigation, the incidence of diabetes was 11.6%, and diabetes as a chronic multiple disease has gradually become the key public health issues of global concern, about 90% of type 2 diabetes.

The incubation period for type 2 diabetes typically lasts more than 10–15 years. During this time, fat accumulation and weight gain in adipose tissue have gradually increased in most people due to overeating and/or lack of exercise. When fat accumulation in adipose tissue reaches a certain level, it will accumulate in non-adipose tissue such as liver and skeletal muscle, which is called heterotopic fat accumulation.

Insulin sensitivity may be reduced when ectopic fat accumulation occurs. In other words, the accumulation of ectopic fat leads to the eventual formation of insulin resistance. Insulin resistance is a condition in which normal doses of insulin produce less than normal biological effects, in which tissue sensitivity to insulin decreases and compensatory insulin secretion by βcells of the pancreas increases, resulting in hyperinsulinemia. Its essence is the insulin-mediated reduction in cellular glucose metabolism. In the early stage of insulin resistance, the body can maintain blood glucose at the normal level by increasing the secretion of compensatory insulin. With the decline of the function of β cells in the pancreas, glucose homeostasis will be damaged when sufficient insulin cannot be produced, and glucose tolerance will be reduced, leading to the occurrence of type 2 diabetes. Insulin resistance is one of the markers of T2D and is associated with low systemic inflammation, characterized by upregulation of cytokines and activation of inflammatory signaling pathways. Involving T1D and T2D, IL-1 is an important inflammatory cytokine. IL-1-mediated cell destruction leads to T1D.

32.3.1 Type 1 Diabetes Mellitus and Autophagy

Type 1 diabetes is a metabolic disease that arises from autoimmune destruction of β cells, characterized by lymphocytic infiltration of pancreatic islet cells. Recent studies have shown that cognitive deficits, such as impaired learning, memory, problem-solving, and thinking flexibility, are more common in T1D subjects than in the general population. In addition, studies have shown that type 1 diabetes increases mitochondrial debris by increasing DRP1 phosphorylation. Type 1 diabetes increases mitochondrial production. Type 1 diabetes increases tau protein phosphorylation. INS and IGF have been shown to protect neurons from cytotoxicity. Insulin reduces autophagy and tau phosphorylation by inhibiting glycogen synthase-mediated kinase-3 (GSk-3) and through the phosphoinositol 3-kinase PI3-k)/Akt signaling pathway (Hakonarson et al. 2007; Santos et al. 2014).

32.3.2 Type 2 Diabetes Mellitus and Autophagy

It is well known that β cell decline and insulin resistance together determine the progression of type 2 diabetes. Insulin inhibits autophagy. It has recently been found that autophagy is inhibited in the liver of mice with insulin resistance/hyperinsulinemia induced by a high-fat diet, and activation of the basic akt-dependent insulin signaling pathway in vivo is accompanied by decreased autophagy levels in the liver. The ratio of LC3-II/LC3-I in the liver of insulin-resistant/hyperinsulinemic mice decreased, but some long-lived macromolecules such as P62 and ubiquitin-like proteins were increased. The transcription levels of several autophagy genes in the liver of these

mice, such as Ulk2, Vsp34, Agt12, and gabarapl1, were also decreased. In contrast, insulin-deficient mice increased the level of autophagy in the liver. In vitro cultured insulin-resistant hepatocytes, autophagy is also inhibited by an insulin-akt dependent manner. In addition, the researchers also found that the liver cells in the insulin inhibition of autophagy are dependent on FoxO1. It is worth noting that this phase consists of two stages: stage of blood glucose levels normal fasting plasma glucose (3.9–6.1 mmol/l) (70–110 mg/dl), and pre-diabetes stage (6.1–6.9 mmol/l fasting glucose (110–125 mg/dl)). In patients with pre-diabetes, fasting blood glucose levels have exceeded the normal range, but are not high enough to diagnose diabetes. The increase of blood glucose level itself indicates that the insulin secretion in pre-diabetes has begun to be relatively insufficient, which means that the insulin-mediated autophagy inhibition in pre-diabetes patients may have begun.

In addition, under specific conditions, oxidative stress can activate autophagy, and oxidative stress plays a necessary role in the mediation of insulin resistance by ectopic fat accumulation. Therefore, in theory, the oxidative stress leading to insulin resistance in patients with insulin resistance can activate autophagy. Two relative forces may coexist to regulate autophagy in turn: excessive nutrients and insulin inhibit autophagy and activate autophagy in the presence of oxidative stress. The final level of autophagy activity depends on the balance of these two forces and changes with the fluctuation of these two forces at different time points or stages. In addition, the role of altered autophagy activity in the development of insulin resistance needs to be further studied, which is an important research content of type 2 diabetes and many metabolic diseases.

The research on autophagy in type 2 diabetes is at a very preliminary stage, and there are few reports on it, and its role is still inconclusive. Theoretically, autophagy activity in type 2 diabetes should gradually increase compared with early diabetes due to insulin deficiency, and the increase in autophagy activity in diabetes has been confirmed by some studies. However, since patients with type 2 diabetes, especially in the early stage of type 2 diabetes, are also accompanied by hyperinsulinemia, it is possible to reduce autophagy activity even if the insulin level is insufficient to overcome insulin resistance to control the blood glucose level. Some reports also support the possibility.

Hyperglycemia, insulin resistance, and relative insulin deficiency are characteristics of diabetes. Glucose is the main substance that induces β cells of the pancreas to secrete insulin. In the case of excessive intake of nutrients, in order to maintain blood sugar levels in the normal range, the body's insulin secretion will increase. During this process, insulin resistance gradually develops. Blood sugar levels are higher than normal only when insulin secretion becomes insufficient to overcome insulin resistance. In other words, the functional threshold of insulin signal is very high. No matter how severe the insulin resistance is, as long as there is enough insulin secretion, the blood glucose can be kept at a normal level. It is now clear that akt-dependent classical insulin signaling pathways are involved in maintaining normal blood glucose levels. MTORC1 also promotes growth by promoting the transformation of glucose metabolism from oxidative phosphorylation to glycolysis. MTORC1 increases the expression of glycolytic enzymes such as PFK (phosphofructose kinase)

driven by HIF1, a translation transcription factor. In addition, mTORC1-dependent activation of SREBP led to an increase in the PPPS flux, which generated NADPH and other intermediate metabolites required for proliferation and growth by utilizing the carbon in glucose. An important early step in autophagy is to activate (unc-51-like kinase 1) ULK1, a kinase that is formed by ATG13, FIP2000, and ATG101, and drives autophagosome formation. Under the condition of adequate nutrition, mTORC1 phosphorylates ULK1, thus preventing its activation by the key activator AMPK, namely, autophagy inhibition. In the case of starvation, mTORC1 dissociates from ULK1, and AMPK binds ULK1 and phosphorylates ULK1, thus activating autophagy (Egan et al. 2011). Therefore, the relative activity of mTORC1 and AMPK in different cell environments largely determines the degree of autophagy induction. MTORC1 by phosphorylation and nuclear translocation inhibition of transcription factors TFEB part regulate autophagy; TFEB drive lysosome biological gene expression and autophagy mechanism. Insulin through the activation of PI3K/Akt pathway, Akt phosphorylation can be directly PRAS40, causes mTORC1 activation, and thus promotes autophagy inhibition. AMPK can increase the LC3 autophagy-related factors and gene expression of BNIP3. Hypoxic activation of REDD1 protein leads to the inactivation of mTORC1 and induces autophagy. Hypoxia also induces autophagy by regulating hypoxia induction factor-1 (HIF-1) or FOXO3 by transcribing NIX/BNIP3L and BNIP3. Mitochondrial ros and AMP-activated protein kinase and ULK1 autophagy signal cascade (Bellot et al. 2009). Activation of inflammatory bodies in hematopoietic cells impairs insulin signaling pathways in several target tissues, thereby reducing glucose tolerance and insulin sensitivity.

TNF induces serine phosphorylation of IRS1 (insulin receptor substrate), leading to impaired signal transduction between the insulin receptor and the downstream PI3k-AKT pathway, leading to insulin resistance.

Insulin promotes many activities related to metabolism and cell growth. For example, insulin stimulates the anabolism of major nutrients (glycogen, protein, and triglycerides). Inflammation can increase insulin resistance. The major cells involved in inflammation and insulin resistance are fat cells. Insulin regulates the uptake of glucose by fat cells and the storage of triglycerides. Different adipocytokines, especially leptin, adiponectin, and endoliptin, can cause dysfunction in pancreatic cells. Adipose tissue also secretes dipeptidine-4 (DDP-4), which promotes the degradation of glucagon-like peptide-1 (GLP-1) and has an insulin-promoting effect on cells (insulin-secreting cells) (Rohrborn et al. 2016). The cytokines including tumor necrosis factor α (TNFα), interleukin 1β (IL-1β), and urothelial (IFN-ɣ) destructs β cells, and regulates the intracellular calcium so insulin release. TNF-α acts on cells, causing them to die more rapidly.

Since the accumulation of fat is directly related to the progression of insulin resistance, the process of fat synthesis is of particular concern. Fatty acids induced by a high-fat diet can activate NLRP3 inflammasomes in macrophages through the Ampk-autophagy-Ros signaling pathway, and the release of IL-1 causes fatty acids to block normal insulin signal transduction in multiple insulin target tissues, eventually leading to insulin resistance (Wen et al. 2011). AMPK inhibits the production of ROS by inhibiting the expression and function of nicotinamide adenine dinucleotide

phosphate (NADPH) oxidase. In addition, insulin is a growth factor that plays its growth-promoting role mainly through the activation of ERK 1/2 MAKP. In addition to inhibiting gluconeogenesis, insulin also inhibits the decomposition of many other substances such as lipolysis, amino acids, and oxidation of fats. And insulin is known to reduce levels of the insulin receptor itself. It has recently been found that long-term action of insulin can inhibit mitochondrial production.

P53 involves a number of steps in glycolysis, including the transport of glucose into cells. For example, the transport of glucose is inhibited by directly inhibiting the transcription of the glucose transporters GLUT1 and GLUT4 or indirectly through the downregulation of GLUT3 expression by inhibiting NF-kB. The transport of glucose is inhibited by direct induction of ras-related glycolysis inhibitors and calcium channel regulator (RRAD) that inhibit the translocation of GLUT1 on the plasma membrane. RRAD overexpression inhibits glucose uptake by muscle and fat cells. P53 also inhibits glycolysis by indirectly inhibiting GLUT4. Insulin-induced insulin receptor activation triggers the translocation of glucose transporters from the intracellular vesicles into the plasma membrane, which in turn triggers a rapid increase in glucose uptake. P53 inhibits the insulin receptor promoter (INSR), thereby indirectly inhibiting glucose uptake by downregulating the insulin receptor (Itahana and Itahana 2018).

It has been reported that p62 is involved in autophagy regulation of brain-derived neurotrophic factor (BDNF), which causes diabetic neuropathy, and renal epidermal growth factor receptor (EGFRs), which has recently been shown to cause diabetic nephropathy.

ROS can induce autophagy by inhibiting mTOR pathway, increasing Beclin1 expression, and transforming lc3-i into lc3-ii. In addition, ROS can act as a signaling molecule to activate JNK-1. The excessive production of ROS results in the opening of mitochondrial permeability transformation pore and the subsequent destruction of mitochondrial membrane potential, leading to the autophagy mediated by PINK1/parkin. PINK1 is a mitochondrial serine/threonine protein kinase, which is detected at a very low level in the mitochondrial intima of normal cells. The loss of mitochondrial membrane potential leads to the increase of PINK1 level and the stabilization of PINK1 in the mitochondrial outer membrane. This results in the production of Parkin and PARK2 genes, which are involved in protein degradation, mitochondrial membrane recruitment, and mitotic induction. The increase of ROS promotes the transcription of BNIP3 NIX, TIGAR, LC3 BNIP3, and p62 by activating transcription factors HIF-1, p53, FOXO3, and Nrf2, respectively (Itahana and Itahana 2018). HIF-1 increases the expression of BNIP3 and NIX/BNIP3L leads to dissociation Beclin1/Bcl2 complex leading to autophagy induction. Thus, HIF-1 can be induced to reduce ROS levels by eliminating impaired mitochondrial autophagy (Bellot et al. 2009).

PERK pathway induces autophagy through ATF4 and stimulates the transcription of ATG5, ATG7, and ATG10. PERK pathway may also play a role in the transformation from lc3-i to lc3-ii. IRE1 and is involved in the activation and enhancement of autophagy inducible beclin-1 transcription by xbp-1, another participant of the IRE1 pathway. ATF6 indirectly participates in autophagy by inducing xbp-1 transcription.

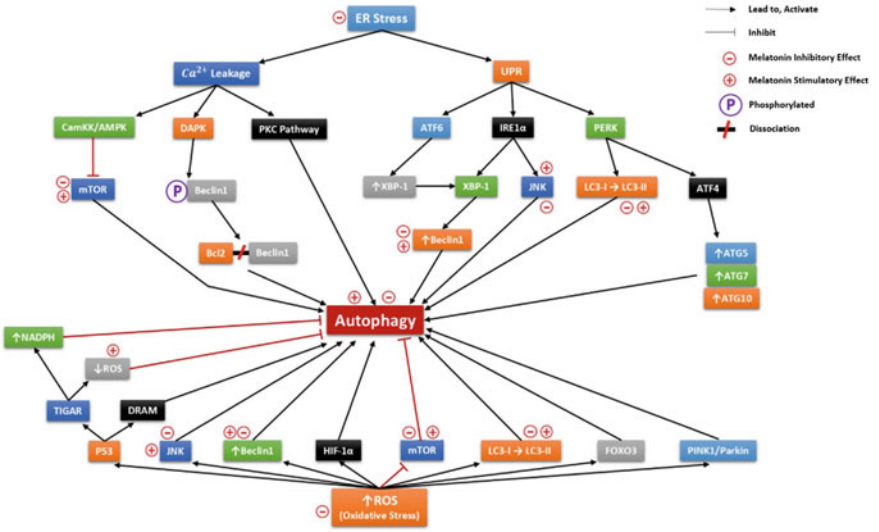

Fig. 32.2 Excessive Ca^{2+} leakage into the cytoplasm leads to autophagy induction through three different mechanisms

Excessive Ca^{2+} leakage into the cytoplasm leads to autophagy induction through three different mechanisms: (a) stimulation of the CamKK/AMPK-dependent pathway leads to inhibition of mTOR; (b) activation of DAPK causes bcl2-beclin1 to be dissociated by Beclin1 phosphorylation; and (c) activation of PKC pathway. Oxidative stress induces autophagy by inhibiting mTOR, increasing Beclin1 expression, transforming LC3-i into LC3-ii, and activating JNK (He et al. 2012) (Fig. 32.2).

32.3.3 Autophagy and Diabetic Complications

Complications of diabetes can be classified as acute and chronic, as well as microangiopathy and macroangiopathy.

32.3.3.1 Autophagy and Diabetic Cardiomyopathy

A decrease in autophagy has been reported in the hearts of many type 1 diabetic mice. Cardiac autophagy in type 2 diabetes has been reported to decrease, increase, or remain unchanged. Studies have shown that cardiac autophagy is inhibited in obesity and metabolic syndrome induced by high-fat diets, but activated in insulin resistance and hyperglycemia induced by fructose and fat diets. These inconsistent results suggest the complexity of autophagy regulation in the progression of diabetic cardiomyopathy. Studies have shown that autophagy in muscle cells,

including cardiomyocytes, is activated by FoxO3, and that insulin inhibits foxo3-mediated autophagy. It has recently been found that basal levels of the classical AKT-dependent insulin signaling pathway are increased in the skeletal muscles of insulin-resistant/hyperinsulinemia mice. Therefore, in people with insulin resistance, under the pressure of hyperinsulinemia, insulin can inhibit the autophagy of cardiac myocytes, which is the same as the autophagy inhibition caused by Atg5 deficiency, resulting in cardiac hypertrophy. This may explain why patients with insulin resistance/hyperinsulinemia often have hypertrophy of the heart muscle.

The effect of Tax1 binding protein 1 (TAX1BP1) on autophagy suggests that Tax1 may be involved in the progression of diabetic cardiomyopathy. TAX1BP1 expression was significantly decreased in stz-induced diabetic mice. TAX1BP1 overexpression in the heart alleviates stz-induced cardiac remodeling in diabetic mice. TAX1BP1 increases autophagy by activating the atypical NF-κB RelB signal. In addition, the use of autophagy inhibitor 3-ma eliminated the protective effect of TAX1BP1 in vivo (Xiao et al. 2018). In the pressure overload mouse model, beclin 1, an autophagy gene, is a heterozygote and can protect the systolic function of the heart rather than damage it. In addition, beclin 1 heterozygote can reduce the area of myocardial infarction induced by ischemia/reperfusion. Controversially, other studies have shown that beclin1 overexpression reduces cell damage, while Atg5 heterozygote overexpression in ischemia/reperfusion models increases cell death. Importantly, interventions that aggravate ischemia or heart failure (e.g., β adrenergic receptor agonists) inhibit autophagy activity in the heart of patients, whereas mitigations (e.g., β adrenergic receptor antagonists) enhance autophagy activity. Another study found that decreased autophagy activity in the heart of diabetic rats was accompanied by increased mitochondrial autophagy activity (Russo et al. 2012).

32.3.3.2 Autophagy and Diabetic Nephropathy

Diabetic nephropathy (DN) is a chronic inflammatory disease in which macrophages are involved in the inflammation and fibrosis of DN, mainly presenting the M1 phenotype. The imbalance of macrophage phenotypic activation of M1/M2 is the key to diabetic nephropathy (DN) (Zhao et al. 2017). The expression of LC3 and the co-localization of LC3 and mitochondrial fluorescent probes were decreased in the renal tubular epithelial cells stimulated by high glucose, and the renal tubular apoptosis was increased (Zhan et al. 2015). In addition, podocytes are the key structure of the glomerular filtration barrier. Damage and loss of podocytes seriously damage the integrity of the glomerular filtration barrier and promote glomerular sclerosis. Therefore, changes in podocyte phenotype and function accelerate the progression of diabetic nephropathy. In diabetic rat models, mTOR activation regulates the expression of LC3II, ribosomal S6 kinase 1 (S6K1) and other proteins, inhibits the formation of autophagosomes, and leads to podocyte injury (Huang et al. 2017; Tremblay et al. 2005). Studies have confirmed that renal tubular epithelial cells are novel insulin-sensitive cells, and renal tubular insulin resistance plays a key role in the pathogenesis of DN. Autophagy inhibits insulin resistance and plays a protective role in DN. Under

the basic conditions, the autophagy activity of proximal tubule cells was maintained at a very low level. Excessive autophagy can lead to damage to renal tubules.

32.3.3.3 Autophagy and Diabetic Retinopathy

Diabetes retinopathy (DR), a microvascular complication of diabetes mellitus (DM), is a major cause of vision loss worldwide. There are two types of DR: (a) non-proliferative diabetic retinopathy (NPDR), which is characterized by microaneurysm and possible intracavitary hemorrhage, hard exudate, and cotton plaque; (b) proliferative diabetic retinopathy (PDR) is characterized by neovascularization of the fundus and iris in addition to vitreous hemorrhage. Under the condition of hyperglycemia, the adhesion of white blood cells to retinal capillaries leads to endothelial damage, and the increase of vascular permeability leads to macular edema and capillary occlusion. Due to capillary occlusion, retinal ischemia stimulates the production of vascular endothelial growth factor (VEGF), which promotes angiogenesis and the generation of PDR. Recent studies have emphasized the role of autophagy in the pathogenesis of diabetic retinopathy. Autophagy has been shown to be associated with an increase and decrease in DR (Dehdashtian et al. 2018; Falavarjani et al. 2015).

32.3.3.4 Autophagy is Associated with Diabetic Neurological Disease

Neurodegenerative changes in central nervous system (CNS) are more and more common in most diabetic patients. Autophagy dysfunction has been implicated in the pathogenesis of many neurodegenerative diseases. T2D patients are at greater risk for vascular dementia and Alzheimer's disease, brain shrinkage, and cognitive impairment (Jayaraman and Pike 2014). R. Noriega-cisneros et al. reported the elevated level of nitric oxide (NO) in the brain mitochondria of diabetic rats induced by streptomycin (STZ) (Zhao et al. 2013). Neurons produced excessive NO to high glucose, which resulted in s-nitrification of ATG4B.ATG4B; s-nitrification can be observed in the central nervous system of diabetic rats with GK hyperglycemia. Studies have shown that s-nitrification produces neurotoxicity by affecting the formation of autophagosomes (Li et al. 2017).

In addition, after spinal cord injury in diabetic patients, autophagy increased, apoptosis increased, and the recovery of motor ability was delayed. The results showed that the stimulation of autophagy could inhibit the apoptosis of diabetic SCI rats, which reduce nerve damage and promote the recovery of motor ability (Zhou et al. 2015).

32.4 Conclusion

Autophagy plays a very complex and important role in glucose homeostasis and lipid balance, affecting glucose metabolism, insulin resistance, oxidative stress, and lipid storage in different tissues, lipoprotein metabolic pathways, etc. The study of autophagy in glucose metabolism is still in a very preliminary stage. The effect of changes in autophagy activity on the development of insulin resistance is an important research content in diabetes and many metabolic diseases. In addition, autophagy regulates the lipid balance of obese people through the role of fat tissue and liver, which not only affects the storage and metabolism of triglycerides, but also affects lipid metabolism through lipoprotein assembly. Therefore, it is still worth further exploration of the deeper mechanism of oxidative stress induction of insulin resistance to autophagy, downregulation of autophagy to change the normal process of adipose tissue differentiation, and whether there will be corresponding complications to the body.

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Chapter 33
Autophagy and Obesity-Related Reproductive Dysfunction

Tao Tao and Huanbai Xu

Abstract Polycystic Ovary Syndrome (PCOS) is a common obesity-related reproductive disease in women of child-bearing age,which is usually accompanied with endocrine and metabolic abnormalities such as hyperandrogenemia and hyperinsulinemia. The abnormal reproductive function of PCOS is mainly characterized by the morphological and functional changes of ovary. Autophagy is involved in the maintenance of human ovarian physiological function as well as in the process of luteal degeneration, and affects the survival of granulosa cells. This chapter introduces the latest research progress of the relationship between autophagy and PCOS. How autophagy is involved in the occurrence and development of PCOS remains to be further studied.

Keywords Autophagy · Polycystic ovary syndrome · Ovarian function

33.1 Characteristics of Polycystic Ovarian Syndrome

With the rapid economic development, the incidence of obesity has increased significantly, and the incidence of Polycystic Ovary Syndrome (PCOS), the best model of endocrine-related reproductive diseases, has also increased significantly. PCOS is a common endocrine and reproductive dysfunction disease among women in the reproductive period, accounting for 5–10% of women. In recent years, studies have shown that in addition to the traditional hyperandrogenemia, hyperinsulinemia also produces extremely important reproductive and metabolic abnormalities in organs such as Hypothalamic-Pituitary-Ovarian (HPO) axis, adrenal gland, pancreas, and

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adipose tissue. In the hypothalamic–pituitary–ovarian axis relationship, the abnormal reproductive function of PCOS is mainly characterized by the changes of ovarian morphology and function, which is the major treatment goal of patients with reproductive dysfunction. The change of ovarian function is the manifestation of abnormal function of different parts of follicle in endocrine and morphology. The root cause of the increase in the number of presinus follicles and the subsequent stasis of sinus follicles is the increased reactivity of ovarian tissue (theca cells, granulosa cells, stroma cells) during the gonadotropin independent phase. Compensatory hyperinsulinemia can aggravate ovarian dysfunction. Abnormal glucose metabolism in PCOS ovaries and high sensitivity caused by hyperinsulinemia/insulin resistance are the key cause to ovarian dysfunction. Autophagy is not only present in ovarian cells, but also affected by insulin and glucose metabolism, etc. Therefore, the further study and understanding of autophagy may provide a possibility for reasonable regulation of ovarian function.

33.2 Autophagy and Maintenance of Ovarian Function

Autophagy is an important mechanism to maintain the physiological function of human ovary. It has been suggested that Autophagy related 7 (ATG7) gene knockout in germ cells may lead to primary ovarian dysfunction (Song et al. 2015). Autophagy of ovarian cells has been found in many animals including fruit flies, fish, quail, primates, mice, and other small animals (Kankuan et al. 2017), suggesting that the autophagy process of ovarian cells in different animals is highly conserved, such as follicular atresia and luteal degeneration. Autophagy is essential for the survival of germ cells during development before the formation of primordial follicular pools. Follicular atresia occurs during each menstrual cycle if the follicle does not ovulate. At first it was thought that follicular atresia was caused by apoptosis. However, recent studies have found that ovarian granulosa cell death is associated with autophagy activated by oxidized low-density lipoprotein (oxLDL)-dependent receptor-1 (LOX-1), suggesting that programmed cell death in the form of autophagy is also involved. oxLDL stimulates the level of Reactive Oxygen Species (ROS) by LOX1, leading to oxidative stress and apoptosis. The study found that the level of oxLDL increased significantly in obese women (Roche et al. 2016), and the incidence of autophagy of ovarian granulosa cells was higher, which may be related to the higher infertility rate in obese women. In young women of normal weight, low ROS levels induce autophagy repair cells in order to avoid apoptosis and increase cell survival. The increase of ROS level in ovarian granulosa cells of elderly women and the decrease of autophagy marker (LC3-II) suggest that with the increase of age, the level of autophagy repair cells decreases correspondingly, leading to the apoptosis of granulosa cells. This explains the decline in fertility among older women. Studies have found that both nutritional deficiency and smoking can induce autophagy cell death in animal ovarian granulosa cells, suggesting that female smoking is closely related to infertility.

33.3 Autophagy and PCOS Pathogenesis

After ovulation, granulosa cells of the ovary become luteinized, and the formation of corpus luteum in vivo. If there is no pregnancy, corpus luteum degenerates after the end of menstruation. According to electron microscopy, autophagy is involved in the process of corpus luteum degeneration, and many cells produce autophagosomes. Molecular studies have found that a specific endocrine voltage activates sodium channels to induce cell autophagy from downstream signals during luteal degeneration in the human ovary, but related signaling pathway studies suggest that ERK 1/2 activation during luteal degeneration is the initiation of luteal cell autophagy. The activation of this signal pathway is independent of the mTOR signal. On the contrary, Beclin-1 expression increased in luteal cells during pregnancy or in the presence of luteal persistence under pathological conditions, suggesting that autophagy affects the survival of granulosa cells. The latest study observed autophagy enhancement in ovarian tissue during follicular phase in PCOS patients and DHEA-induced PCOS rats by transmission electron microscopy: in the experiment, autophagy labeled protein Light Chain 3B (LC3B) of PCOS rat ovarian granulosa cells increased, while the level of autophagy substrate Sequestosome1 (SQSTM1)/p62 showed a downward trend. The LC3-II/LC3-I ratio of ovarian tissue in PCOS patients was significantly higher than that in normal ovarian tissue. Regression analysis suggested that genes such as EGFR, ERBB2, FOXO1, MAPK1, NFKB1, IGF1, TP53, and MAPK9 may be related to autophagy activation of PCOS ovarian tissue (Li et al. 2018). The activation of EGFR can inhibit the meiosis of oocytes and granulosa cells through the expression of related factors in the downstream signaling pathway, thus hindering the maturation of follicles. The phosphorylated ERK 1/2 level was decreased in granulosa cells from PCOS patient, while FOXO1 expression was significantly increased in the cumulus cells of PCOS patients. The increased expression of ERK 1/2 can promote the proliferation of granulosa cells and affect follicular development. NF-κB1 is involved in the regulation of transcription of genes involved in pathophysiological processes such as inflammation. Recent studies using zebrafish in vitro experiments have found that IGF-1 may play a role in the differentiation and development of primary follicles. Compared with the control group, the increased level of IGF-1 increases the number of primary follicles (Katti et al. 2017), and IGF-1 may be an important factor in follicular development and PCOS. Interestingly, the PI3K/AKT/FOXO1/mTOR signaling pathway is involved in the interaction among androgen, insulin, IGF-1, and high-sugar diet in patient with acne (Ju et al. 2017). Recent studies have examined the levels of mRNA produced by transcription of autophagy-related genes (ATG3 / 5/13/14, BECN1, GABARAP, RB1CC1, SH3GLB1, SQSTM1) and found that the expression of autophagy-related genes was down-regulated in the endometrium, suggesting that increased androgen levels in PCOS patients are related to the down-regulation of endometrial autophagy genes, but this process can be partially reversed by metformin (Sumarac-Dumanovic et al. 2017). However, the role of autophagy in the normal endometrial cycle in women and in the pathogenesis of PCOS patients still needs to be further confirmed.

33.4 Conclusion

Autophagy plays an important role in the maintenance of ovarian function. Autophagy is a double-edged sword and plays a completely opposite role in the dynamic evolution of corpus luteum. At present, studies on autophagy and the pathogenesis of polycystic ovary syndrome are still lacking. At present, it is currently recognized that PCOS has two major pathological cores: hyperinsulinemia and hyperandrogenemia, the role of autophagy in the occurrence and development of PCOS still needs to be further studied.

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Part VII
Autophagy and Kidney Diseases

Autophagy is an "auto-digestive" process in a cell that promotes the delivery of intracellular components such as damage organelles, from the cytoplasm to lysosomal or vacuolar compartments for terminal degradation and recycling. There are so many factors (hypoxia, growth factor deprivation, and so on) which can induce autophagy in vivo and in vitro. It has been identified over 30 genes involved in autophagy in yeast, and they have been termed autophagy-related genes (Atgs).

In the past decade, so many people have an intense interest in elucidating the basic molecular mechanism of autophagy and defining its roles in human health and disease. It has been shown that autophagy deficiency results in the accumulation of protein aggregates in various organs including the central nervous system, liver, and kidneys. The kidney is one of the most complicated organs in the body, physiologically, structurally, and metabolically. At present, in developed and developing countries, the number of patients with kidney diseases is increasing and accounts for a significant portion of medical expenses. Unfortunately, most kidney diseases are incurable. Under stress conditions, including hypoxia, genotoxic damage, oxidative stress, and ER stress, autophagy is activated and plays a critical role in kidney cells' survival.

In renal systems, it has been shown that autophagy plays important roles in the maintenance of renal function under both physiological and pathological conditions. It is reported that autophagy plays a helpful role not only in acute kidney injury, such as ischemic reperfusion injury, but also in chronic disorders such as diabetic nephropathy.

We give a general review of the current understanding of the role and regulation of autophagy in acute kidney injury (AKI), glomerular diseases and diabetic nephropathy.

Chapter 34
Autophagy and Acute Kidney Injury

Jing Cui, Xueyuan Bai, and Xiangmei Chen

Abstract Acute kidney injury (AKI) is one of the major kidney diseases associated with poor clinical outcomes both in short- and long-term, which caused by toxins, transient ischemia, and so on. Autophagy is a cellular stress response that plays important roles in the pathogenesis of various diseases, including kidney diseases. Autophagy is induced in proximal tubules during AKI. It has been demonstrated that autophagy plays a renoprotective role in AKI by pharmacological and genetic inhibitory studies. However, the role of autophagy in kidney recovery and repair from AKI remains unknown mostly. In many studies, a dynamic change of autophagy was important for tubular proliferation and repair in the recovery phase of AKI. Moreover, autophagy may not only promote renal fibrosis through inducing tubular atrophy and decomposition but also prevent it by mediating intracellular degradation of excessive collagen in terms of renal fibrosis. In further researches, we expect to clarify the regulation of autophagy in kidney injury and repair, and find out therapeutic drugs for treating AKI and preventing its progression to chronic kidney disease.

Keywords Reactive oxygen species · Autophagy · Acute kidney injury · Ischemia-reperfusion

34.1 Basal Autophagy in the Tubular Epithelium in the Kidney

In a wide type of animals, the level of constitutive basal autophagy observed in proximal tubular cells is high. For example, if this autophagy is inhibited in proximal tubule-specific ATG5 knockout mice, then the animal develops interstitial fibrosis and renal failure with aging which proved that autophagy is indispensable in this part of the kidney (Kimura et al. 2011). On the other hand, in the distal tubule and the

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collecting duct, when the autophagy is inhibited, there are not obvious pathologies or histological changes in the mouse without added insults (Kume et al. 2010).

34.2 Autophagy and Cell Death in Toxic Renal Injury

Nephrotoxic medications and heavy metals, such as cisplatin, cyclosporine, arsenic, and cadmium mainly target the proximal tubules in the kidney and up-regulate autophagy in tissue culture experiments and murine models of toxic renal injury. In the first hours of injury in models using cisplatin, cyclosporine, heavy metals, or aristolochic acid, autophagy is activately measured by LC3-II accumulation and visualization of autophagosomes (Zeng et al. 2014).

34.2.1 Effect of Nephrotoxic Drugs on Autophagy Activity in Acute Kidney Injury

Among many toxins, cisplatin is the most widely studied and a lot of the data presented is derived from experiments using this agent. We still are not sure how toxic injury causes autophagy induction, but there are several candidate pathways. For example, oxidative stress caused by toxins could be the main inducer. It has been improved that cisplatin induces cell death via both heme oxygenase 1 and autophagy. The lack of this protein induces ROS and tubular apoptosis suggesting that this important protein regulates autophagy (Bolisetty et al. 2010). The main toxic effect of cisplatin is DNA damage. It has been acknowledged that DNA damage is followed by p53 nuclear translocation resulting in the induction of downstream target genes that regulate cell cycle progression and initiation of apoptosis. In addition, when the p53 is inhibited, the autophagic response is hindered in the renal tubular cells. The fact suggests that p53 not only regulates cell death but also autophagy (Kenzelmann Broz et al. 2013). There are more renal damages including renal dysfunction, tissue damage, and cell death by apoptosis in renal proximal tubule-specific Atg7-knockout mice caused by cisplatin. The result may suggest that autophagy is protective against toxin-induced injury. Consistently, rapamycin can induce autophagy up-regulation and ameliorate the damage caused by cisplatin and gentamicin. However, when autophagic pathway is overwhelmed, there is nothing to stop apoptosis.

Metformin is one of the most common prescriptions for patients with type 2 diabetes. It has been reported that the drug can protect the kidney from gentamicin-induced nephrotoxicity. In one study, people injected cisplatin into intraperitoneal to induce acute kidney injury model. In cisplatin group, the mice exhibited severe histological damage and kidney dysfunction. In metformin group, they found metformin could improve cisplatin-induced acute kidney injury. Metformin may

inhibit cisplatin-induced tubular cell apoptosis and AKI through promoting AMPKα activation and autophagy induction in the tubular cells (Li et al. 2016).

34.2.2 Regulation of Autophagy on Inflammasome in Acute Kidney Injury

The NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome plays an important role in renal inflammation and fibrosis. People have evaluated the role of inflammasome-independent NLRP3 in renal tubular cells and assessed the value of NLRP3 as a therapeutic target for acute kidney injury. In this study, hypoxia increased significantly the expression of NLRP3, which was independent of ASC, caspase-1, and IL-1β. They found that hypoxia relocalized NLRP3 from the cytosol to the mitochondria in renal tubular cells and NLRP3 was bound to mitochondrial antiviral signal protein (MAVS). In renal tubular cells under hypoxia, the deletion of NLRP3 or MAVS weakened ROS production and depolarization of the mitochondrial membrane potentials. Mitophagy was upregulated in NLRP3 KO kidney relative to the baseline and attenuated the acute kidney injury. The results indicated that inflammasome-independent NLRP3 in renal tubular cells is important in mitochondrial ROS production and injury by binding to MAVS in hypoxic injury. Importantly, we speculate inflammasome-independent NLRP3 could be a therapeutic target of AKI to prevent the progression of chronic kidney disease.

The other study explored the role of the NOD-like receptor family, pyrin domain containing (NLRP3) inflammasome and autophagy in Astragaloside IV (AS IV)-mediated protection against cisplatin-induced kidney injury in rats. In one study, cisplatin induced acute kidney injury and activated the NLRP3 inflammasome. In the AS IV group, people found that the cisplatin-induced injury can be improved and the decrease of NLRP3 and pro-inflammatory cytokines. Moreover, cisplatin inhibited autophagy by the conversion of LC3 II and the expression of p62. AS IV effectively declined cisplatin-induced injury via autophagy inhibition and the decreased NLRP3 expression. It may be a novel therapeutic alleviating the toxic effects of platinum-based chemotherapy (Kim et al. 2018).

34.2.3 Therapeutic Effect of Immunosuppressive Agents on Autophagy in Acute Kidney Injury

Cyclosporine is one commonly used immunosuppressant in clinic, which has similar effects on autophagy as cisplatin including causing ER stress, oxidative stress, and tubular cell death (Yadav et al. 2015). It has known that autophagy alleviates injury both in acute and chronic cyclosporine injury models. In addition, cyclosporine induces hemodynamic changes in the kidney and chronic metabolic stress via effects

on mitochondrial respiration in autophagy-deficient cells. In cyclosporine-induced kidney injury, transmembrane BAX inhibitor motif containing 6 (TMBIM6) regulated autophagy and lysosomal function both in vitro and in vivo. In ischemic and toxic renal injury models, autophagy and apoptosis have very similar roles hence therapeutics that work in one disease might also work in the other.

34.2.4 Effect of Biglycan on Autophagy in Acute Kidney Injury

Biglycan which is a small leucine-rich proteoglycan acts as a danger signal and is classically thought to promote macrophage recruitment via toll-like receptors (TLR) 2 and 4. It is reported that biglycan signaling regulates inflammation through TLR 2/4 and the CD14 co-receptor. It has been shown that a marked increase in the number of autophagic macrophages in mice stably overexpressing soluble biglycan. CD44 is a receptor involved in adhesion, migration, lymphocyte activation, and angiogenesis. In vivo, transient overexpression of circulating biglycan enhanced M1 macrophage recruitment into the kidneys. The biglycan-CD44 interaction increased M1 autophagy and the number of renal M2 macrophages and reduced tubular damage following IRI. The above experiment demonstrated that CD44 is a novel signaling co-receptor for biglycan, an interaction that is required for TLR4-CD44-dependent pro-autophagic activity in macrophages (Poluzzi et al. 2019).

34.2.5 Effects of Arsenic and Its Compounds on Autophagy in Acute Kidney Injury

Arsenic and its compounds are toxic environmental pollutants and known as carcinogens. In one study, scientists investigated the mechanism of arsenite-induced damage in renal cells. In human embryonic kidney cells (HEK293), sodium arsenite reduces cell viability in a dose- and time-dependent manner. Arsenite reduces Akt activity and the Bcl2 level but increases caspase 3 activity and the cytochrome c level, which results in the decline of cell viability. PTEN is the upstream negative regulator of Akt activity and was reduced by arsenite. In this study, arsenite activates ERK and JNK, which can increase the levels of LC3 and p62. However, lysosomal degradation activity and autophagic activity were both decreased. The addition of the mTOR inhibitor rapamycin and the knockdown of mTOR expression both activated the autophagic pathway that promoted the removal of damaged proteins, increased ERK activity, reduced JNK activity, and the p62 level in arsenite-treated cells. These results demonstrate the critical role of mTOR in regulating the cell fate of arsenite-exposed renal cells.

34.3 Autophagy in Acute Ischemic Kidney Injury

34.3.1 Acute Ischemic Renal Injury

In clinic, hypovolemia, hypotension, or heart failure cause transient ischemia, which further commonly results in AKI and accounts for nearly one-third of patients requiring acute renal replacement therapy (Havasi and Dong 2016). It is well known that acute ischemia is one common factor in acute kidney injury. The detachment, dysfunction, and death of these cells are the main causes in the pathophysiological and clinical aspects of ischemic AKI, but inflammation may partly mediate duration and long-term consequences of AKI. Ischemia causes some cells to die by various death mechanisms, whereas other cells undergo sublethal injury. PTCs release cytokines and chemokines after cell injury, and these factors affect directly on endothelial function.

34.3.2 The Role of Autophagy in the Development of Acute Ischemic Renal Injury

Dysfunctional autophagy significantly contributes to ischemic renal dysfunction. In numerous experimental systems, including various animal models as well as in humans, ischemia–reperfusion induces autophagy in PTCs. For example, in mice and rats, ischemia increased expression of LC3 and other autophagy-related proteins, also increased the number of LC3-GFP-positive autophagosomes in cell lines especially after treatment with a lysosomal inhibitor that prevented LC3 degradation. People observed increased colocalization between LC3 and a lysosomal protein LAMP2 in vacuolar structures both in human kidney proximal tubular cell line (HK-2) and in intact kidneys after ischemia. Hypoxia induced autophagy and apoptosis in the kidney development via beclin 1, hypoxia-inducible factor 1α (HIF-1α), AKT, and mTOR signaling. The mechanism is still unclear by which ischemia up-regulates autophagy (Molitoris 2014).

It is reported that fibroblast growth factor 10 (FGF10), a multifunctional FGF family member, exerts a protective effect against cerebral ischemia injury and myocardial damage. In one study, FGF10 treatment improved renal function and histological integrity in a rat model of renal I/R injury. It is observed that FGF10 efficiently reduced I/R-induced elevation in blood urea nitrogen, serum creatinine as well as apoptosis induction of RTCs. The investigators found that autophagy activation following I/R was suppressed by FGF10 treatment. The mTOR pathway involved in the process, which was demonstrated by the combined treatment of FGF10 with Rapamycin partially reversed the renoprotective effect of FGF10. FGF10 could improve kidney I/R injury by inhibiting excessive autophagy and inflammatory response and may, therefore, have the potential to be used for the prevention and treatment of I/R-associated AKI (Tan et al. 2018).

Telomeres become shorter with aging, influenced by environmental factors, as well as specific genetic defects in the underlying telomere mechanisms. Telomerase is a reverse transcriptase enzyme complex that adds DNA sequence repeats (TTAGGG) to the 3′ end of DNA strands in the telomere regions at the ends of eukaryotic chromosomes. Telomerase contains major components in the transcriptase ribonucleoprotein complex—the RNA-directed DNA polymerase, TerT, and the RNA template, TerC—which together prevent telomere shortening by adding telomeric DNA repeats to chromosome ends. TerC or TerT gene mutations are invariably associated with marked telomere shortening, resulting in dyskeratosis congenita and inherited bone (Harris and Cheng 2016).

In either TerT or TerC deficiency G4 mice, induction of renal ischemic injury led to significantly delayed recovery compared to wild-type mice. Electron microscopy demonstrated increased autophagosome formation in wild-type mice, with significant delay of autophagosome development in TerC and TerT KO mice. In addition, the increased expression of LC3 II was delayed and the accumulation of P62 was prolonged. Therefore, ischemic kidney injury may impair autophagy in mice with telomerase deficiency. Autophagy plays an important role in the quality of cellular components to prevent cell senescence. Meanwhile, senescent cells increase lysosomal enzymes directed toward lipofuscin-rich lysosomes, but effective autophagic degradation activity of these enzymes declined, leaving the lipofuscin non-degradable, which further decreases autophagy in senescent post-mitotic cells In addition, in telomerase-deficient mice, the level of p16 was also increased, and greater activation of the mTOR pathway following ischemic kidney injury. The p16 protein is a member of the INK4 family (p16 INK4a), which is a cyclin-dependent kinase inhibitor that inhibits the cell cycle by blocking progression from G1 phase to S phase. It is well known that it is a robust biomarker and a possible effector in mammalian renal aging. Deletion of p16 results in improved kidney regeneration and decreased capillary rarefaction after I/R. However, Rapamycin partially restored the autophagy response in kidneys following ischemic injury without significant effect on either upregulated p16 or renal tubule epithelial cell proliferation. Therefore, the deletion of the ability to maintain normal telomere length in mice impaired recovery from AKI. The increase of tubule cell senescence and impairment of autophagy may be mediated in part by increased mTOR signaling.

One of the few disease processes where autophagy inhibition seems to be beneficial is AKI due to sepsis. In lipopolysaccharide (LPS) induced-AKI mice, autophagy was induced in the renal cortex that mainly consists of tubules. After autophagy was inhibited, LPS-induced acute kidney injury and inflammation were both alleviated. However, the latest study by Mei et al., showed that pharmacological and genetic suppression of autophagy exacerbates LPS-induced acute kidney injury in mice.

34.4 Autophagy in Response to Sepsis

34.4.1 Sepsis

It is reported that autophagy in rat proximal tubules was transiently induced at 3 h but declined at 9 h until 18 h during cecal ligation and puncture (CLP) model of sepsis. Either temsirolimus or an inducer of AMP kinase, 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), protected proximal tubules and improved renal function by augmentation of autophagy in a mouse model of endotoxemia. In these studies, endotoxemia was associated with activation of mTORC1. Therefore, it is likely that mTORC1 inhibition enhances autophagic flux. In the CLP model, induction of autophagy by AICAR-induced activation of AMPK decreased circulating cytokines, endothelial activation, and improved decline in renal function. Contrary to the accepted paradigm, a recent study has reported that CaMKIV-dependent preservation of mTORC1 is indispensable in LPS-induced autophagy in renal tubular cells and macrophages both in vitro and in vivo. A form of selective autophagy pexophagy was recently shown to be induced in LPS-induced AKI and lysosomal defect accumulated dysfunctional peroxisomes that promoted oxidative injury.

34.4.2 The Role of Autophagy in Sepsis

Acute systemic inflammation, such as sepsis, induces a cytokine storm and severely affects multiple tissues, for example, the kidney. Autophagy harbors the capacity to suppress sepsis-induced kidney injury through regulation of infection and through targeting inflammasome and type I interferon responses. Thus, autophagy hampers key innate immune responses to prevent kidney diseases. Conversely, autophagy can also activate a type I IFN response and promote IL1B secretion. Autophagy plays the pro- and anti-inflammatory roles in inhibiting excessive inflammatory responses, and proper modifications of autophagy can suppress kidney diseases due to systemic inflammation (Havasi and Dong 2016).

Tubular atrophy and interstitial fibrosis are the hallmark of chronic kidney disease, people found that autophagy, apoptosis and necrosis are all augmented in animal models of renal fibrosis using unilateral ureteral obstruction (UUO). Again, autophagy seems to precede apoptosis and interstitial fibrosis in the injury process. When autophagy was inhibited in the UUO model, the cell death was enhanced and renal outcome was worsened. However, autophagy deficiency in proximal tubules suppressed tubular cell death and inflammation, resulting in attenuation of renal fibrosis in UUO. Transforming growth factor-β1 (TGF-β1), a cytokine that has been established as a central mediator of kidney fibrosis by autophagy (Molitoris 2014).

In the kidney injury induced by UUO, a model of progressive kidney fibrosis, autophagy was induced and that deficiency of autophagic protein LC3 leads to increased collagen deposition and mature TGF-b levels in obstructed kidneys. In

this study, autophagy regulates TGF-b expression and suppresses kidney fibrosis. In the obstructed kidneys, the level of GFP-LC3 puncta formation was increased in tubular epithelial cells. The increased expression of beclin 1, LC3-I, and LC3-II proteins were similar with the previous report, in which beclin 1, LC3, and increased autophagic vacuoles were increased in obstructed renal tubules.

Both induction of autophagy and apoptosis have been shown to occur in a time-dependent manner in the UUO model. Indeed, increased cell death caused by apoptosis leading to tubular epithelial loss is a prominent feature in UUO. The findings demonstrated that inhibition of beclin 1 enhanced RTEC apoptosis in the obstructed kidneys. These data suggest that beclin 1 functions to protect RTECs from apoptosis after UUO injury.

It is well known that a hallmark of CKD is tubulointerstitial fibrosis with excessive matrix deposition produced by myofibroblasts. People found that treatment with exogenous TGF-β1 induces autophagy in primary cultured mouse RTECs and HK-2 cells. It is thought that actions of TGF-β1 are potent inducers of ECM production and fibrogenesis, and they are induced during kidney injury, including the actions induced by UUO. One study found that the deletion of LC3 resulted in increased mature TGF-β proteins in the obstructed kidneys after UUO. The previous results suggest that LC3 decreases mature TGF-β levels in RTECs via autophagic degradation, reduces TGF-β secretion, and suppresses the development of interstitial fibrosis induced by UUO (Ding et al. 2014; Xu et al. 2013).

34.5 Inflammation and Acute Kidney Injury

34.5.1 The Role of Inflammation in Acute Kidney Injury

It is reported that the role of autophagy in kidney diseases and aging through genetic modifications of autophagy-related genes. Mitophagy plays a key role in regulating inflammation. It has been thought that multiple immune functions of autophagy are suggested to play key roles in kidney diseases (Havasi and Dong 2016).

It is well known that inflammation is a key pathology in kidney diseases. A lot of acute or chronic factors, such as ischemia, drugs, toxins, metabolism. In turn, the damage of the tubules provokes inflammatory responses and results in kidney fibrosis. Moreover, chronic inflammation is prevalent in patients with CKD, potentially due to chronic infection, or for unknown reasons. Chronic inflammation exacerbates kidney anemia and kidney function, and provokes malnutrition. Glomerular inflammation, called glomerulonephritis, causes proteinuria and worsening of GFR caused by nephron loss. It is reported that glomerulonephritis often accompanies deposits of immune complexes and damage to the glomerular capillaries

Because injuries of tubules mainly occur in proximal tubules, the role of autophagy in tubules has mainly been demonstrated in proximal tubules. Proximal tubules consume a large amount of oxygen and energy for electrolyte reabsorption. There are

abundant lysosomes in kidney tubules which perform endocytosis actively. For these reasons, proximal tubules are rich in mitochondria and lysosomes.

Acute pathological stimuli, such as ischemia/reperfusion injury, toxic side effects of drugs (such as cisplatin and cyclosporine A), and urinary tract obstruction, quickly upregulated autophagy in kidney tubules. Functionally, autophagy largely plays protective roles, and autophagy preserves the kidney from tubular damage and its consequent fibrosis (Kimura et al. 2011).

34.5.2 Autophagy Inhibits Tubular Inflammation by Clearing Damaged Mitochondria

Autophagy targets damaged and depolarized mitochondria and regulates mitochondrial quality control. There are lots of mitochondria in kidney tubules and reactive oxygen species were produced in mitochondria which can trigger an inflammatory response, leading to pathogenesis in kidney disease. Acute insults such as ischemia/reperfusion injury and toxic drugs induce autophagy to prevent cell death. In response to these stimuli, autophagy-deficient kidney cells accumulate abnormal mitochondria.

This is well-characterized in a cisplatin-induced kidney injury model in vitro. Cisplatin, a frequently used chemotherapeutic drug, induces mitochondrial damage and promotes production of ROS. Autophagy improves the damage of kidney tubules by cisplatin through the removal of ROS-producing mitochondria. Autophagy not only removes the ROS-producing acutely damaged mitochondria, but also controls the quality of mitochondria. Lack of quality control of mitochondria in aging autophagy-deficient mice worsens mitochondrial function, which is a characteristic of the aging process.

It is well known that mitochondria are the main intracellular energy source through oxidative phosphorylation. The damaged mitochondrial function in autophagy-deficient kidney affects intracellular metabolism. In autophagy-deficient kidney, mitochondrial respiratory chain activity was reduced due to depolarization, which affects the kidney's adaptation to metabolic acidosis, a common pathological condition seen in patients with kidney diseases. It has been implicated that failure to cope with metabolic acidosis as a pathogenesis of systemic inflammation is seen in chronic kidney disease and malnutrition. Nowadays, people emphasize the link between intracellular metabolism and immune systems (immunometabolism). Regulation of mitochondrial metabolism by autophagy may play immunometabolic roles in kidney diseases.

The scientists established a transgenic mouse with a pH-sensitive fluorescent mitochondrial signal in order to detect mitophagy. The analyses of this mouse, called mito-QC, revealed that the kidney is one of the most active tissues of mitophagy.

It is reported that lysosomal rupture strongly activates inflammation and crystals such as monosodium urate damages lysosomal membranes. Both acute and chronic hyperuricemia cause lysosomal rupture in kidney. As a result, uric acid becomes oversaturated acutely in the urine, leading to the formation of urate crystals in the kidney. Currently, crystal deposits in proximal tubules are supposed to induce hyperuricemia-induced kidney injury. These urinary crystals are endocytosed and delivered to lysosomes, where rupture happens while inducing inflammation.

It is thought that autophagy in kidney also protects against lysosomal rupture-induced inflammatory injuries. Genetic ablation of Atg5 exacerbates kidney dysfunction in acute hyperuricemic kidney injury mice.

DAMPs encompass a range of cellular components, for example, nuclear (such as histones, DNA/RNA, and HMGB1) and cytosolic (mitochondrial DNA, ATP, and glycoproteins) ones. It is reported that DAMPs activate the inflammasome and type I interferon responses, which in turn trigger macrophages and leukocytes to infiltrate into the kidney interstitium to promote inflammation. Autophagy can suppress the release of DAMPs. Endogenous DAMPs are well-known targets for autophagic degradation. Autophagy suppressed the release of mitochondria DNA, which strongly induces inflammasome activation. One of the best-characterized DAMPs secreted by autophagy is IL1β. Whereas basal autophagy suppresses IL1β secretion through clearance of DAMPs such as damaged mitochondria that generate ROS, autophagy promotes IL1β secretion once the inflammasome is activated. Generally, autophagy secretion may play pro-inflammatory roles in kidney disease formation.

In kidney tubular, autophagy largely inhibits inflammation through the removal of damaged and malfunctioning mitochondria. Removal of damaged lysosomes also may suppress DAMPs release.

Tubular epithelial cells are highly dependent on autophagy to maintain homeostasis and respond to stimulants. Autophagy and apoptosis are interconnected and their complexity affords new targets for effective interventions. Though there are not lots of human trials, several therapeutic interventions targeting the apoptotic and/or the autophagic pathway have shown beneficial effects in animal models. Depending on the specific disease process, cell type, the stage of the disease and so on, autophagy can both play a protective or damaging role.

The mTOR inhibitors are the most commonly used autophagy activators. Certainly, autophagy can be also induced by various other ways, by activating AMPK or sirtuin 1, activators of autophagy, or by acetyl coenzyme A depletion. Autophagy inhibition is the target in various cancers because autophagy increases cancer growth.

Indeed, scientists are still far from developing the preferred product profile for a drug which treats AKI by the autophagic pathway, but we already gained crucial knowledge from using certain medications in clinical practice that at least partially target autophagy. For example, rapamycin or sirolimus which can inhibit mTOR has been in clinical practice for years. However, they not only induce autophagy but also alter the function of several other mTOR substrates.

Furthermore, sirolimus plays an important role in worsening proteinuria, glomerulosclerosis, diabetes mellitus, and dyslipidemia. Off-target effects and adverse reactions are the most serious puzzle in most of the currently used autophagy modulating chemicals and the net biological effect might be unfavorable.

Autophagy inhibition would be more beneficial at one point, while autophagy up-regulation might be required at another point. Autophagy plays a complex role in various organs and diseases. Despite many difficulties, we still expect that more specific inhibitors or inducers of autophagy will become available in clinical trials.

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Chapter 35
Autophagy and Glomerular Diseases

Jing Cui, Xueyuan Bai, and Xiangmei Chen

Abstract Autophagy is an endogenous and essential process which maintains cellular homeostasis and directs cell fate. The glomerular diseases are one main part of the kidney diseases, often associated with poor clinical outcomes. The regulation of autophagy contributes to the progression of various glomerular diseases, including focal segmental glomerulosclerosis, lupus nephritis, and so on. For example, it has been demonstrated that prevention of autophagic flux in the kidney epithelium and podocytes is sufficient to trigger a degenerative disease of the kidney with many of the manifestations of human FSGS. We review the roles of autophagy in glomerular diseases. Therapies in clinical use, and in preclinical or clinical development, are also discussed in relation to their effects on autophagy in glomerular diseases.

Keywords Podocytes · Polycystic kidney disease · Inflammation · Focal segmental glomerulosclerosis

It is well known that podocytes are terminally differentiated post-mitotic cells in which autophagy plays an important role in removing toxic, damaged organelles, and proteins. It is known that basal autophagy is helpful for glomerular health, such as glomerular development, podocyte loss, late onset glomerular sclerosis, and accumulation of protein aggregates in aging. Under stress conditions, inhibition of autophagy leads to worsening renal failure in several animal models of glomerular diseases.

35.1 Autophagy and Podocytes

Podocyte dysfunction causes the glomerular disease which results in 90% of end-stage kidney disease. It is the fact that Notch signaling pathway plays an important role in cellular differentiation and organ development. The pathway all display a

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specific cell and tissue type during development. Exactly, Notch1 is located in the renal epithelial including podocyte component of the mouse metanephros. In developing kidney, the level of Notch1 activity is very high in glomerular. In addition, the deletion of Notch2 genes leads to metanephroi without glomerular in developing kidney. Notch3 and Notch4 were respectively mainly detected in the distal portion of the S-shaped body and endothelial cells. Conversely, in the mature kidney Notch1 can be detected at very low activity (Zhang et al. 2017).

Notch signaling is important in the terms of conserved cell–cell communication, which regulates the implementation of differentiation, proliferation, and apoptosis. Thus, it makes a crucial contribution in kidney development including podocyte specification. In early differentiation of podocytes, it has been demonstrated that Notch signaling is very important. The cultivation of mouse metanephroi with csecretase inhibitor DAPT which can block all Notch pathways caused a severe deficiency in glomeruli, which can delay the development of renal epithelia. Meanwhile, Notch pathway seemed to be dispensable for podocytes in an advanced stage of differentiation because molecular of Notch signaling progressively diminished when kidney as well as podocytes became mature. Collectively, Notch pathway is indispensable in podocyte differentiation.

During embryogenesis and post-natal development including podocyte maturation, autophagy plays the important role in specific cytosolic rearrangements needed for proliferation and differentiation. Because of the deficient autophagy, cellular homeostasis was impaired and cellular integrity could not be maintained, even maturation could be delayed. Finally, when autophagy levels were decreased by Notch inhibition, podocyte differentiation was impaired, which was demonstrated by the decline of podocyte differentiation marker nephrin. In the study, they found that nephrin was recovered and DAPT-induced injury was ameliorated. This result demonstrated that the differentiation defect was directly caused by the decreased level of autophagy. Increasing autophagy status can rescue the differentiation when Notch signaling was impaired (Zhang et al. 2017).

35.2 Autophagy and Focal Segmental Glomerulosclerosis (FSGS)

FSGS is a heterogeneous renal disease with typically poor outcome due to progressive loss of kidney function. We still don't clarify the cause of primary FSGS. Over the last 15 years, it has been identified that several mutations in genes expressed by podocytes that code for proteins of the filtration barrier of the kidney (known as the slit diaphragm). Mutations in these genes lead to severe life-threatening kidney disease in the neonatal period. Recently, scientists have found that an HDL lipid transfer protein APOL1 has also been associated with the development of FSGS in patients of African ancestry. Although FSGS is characterized by lesions in the glomerulus, particularly podocytes, and although mutations restricted to podocytes cause FSGS,

people also verify that early functional defects in proximal tubule cells, ranging from the isolated glycosuria to multiple solute wasting. For example, Fanconi syndrome is found in some patients with FSGS. In conclusion, pathologic abnormalities may lie in the tubules as well as glomerular cells (Kawakami et al. 2015).

Investigators prevented normal autophagic pathways by mutating autophagy genes ATG5 or ATG7 during nephrogenesis in mice. In 2-month age, mutant mice developed mild podocyte and tubular dysfunction. The mutant mice developed profound glomerular and tubular changes bearing close similarity to human disease by 4 months, and organ failure by 6 months. Ultrastructurally, in podocytes and tubular cells, it is shown that vacuolization, abnormal mitochondria, and evidence of endoplasmic reticulum stress, features that precede the appearance of histologic or clinical disease, which were similar with human idiopathic FSGS kidney biopsy specimens. In podocytes and tubules of 2-month-old mutant mice, the level of reactive oxygen species, activation of endoplasmic reticulum stress pathways, phosphorylation of p38, and mitochondrial dysfunction are increased. Meanwhile, in cultured proximal tubule cells isolated from mutant mice, the mitochondrial dysfunction and elevated mitochondrial reactive oxygen species generation was suppressed by a mitochondrial superoxide scavenger. In conclusion, mitochondrial dysfunction and endoplasmic reticulum stress caused by impaired autophagic organelle turnover in podocytes and tubular epithelium results in sufficiently many of the manifestations of FSGS in mice.

35.3 Autophagy and Polycystic Kidney Disease (PKD)

Polycystic kidney disease (PKD) is the most common genetic form of CKD and causes about 5% of all end-stage renal diseases. In PKD, it has been demonstrated that healthy kidney tissue is slowly replaced by growing cysts, resulting in renal failure (Havasi and Dong 2016). It has been well known that apoptosis in tubular epithelial cells has a central role in cyst formation. Meanwhile, autophagy might also play a vital role in PKD, potentially by altering apoptosis. Although there are many signaling pathways involved in both autophagy and apoptosis that are dysregulated in PKD, such as the transcription factor, signal transducer and activator of transcription 1 (STAT1), and MAPK8 (JUNK1), it has not been clarified that how the crosstalk between apoptosis and autophagy affects tubular cell fate in PKD. In a recent study, the level of autophagy was lower in the cells with shorter cilia. Conversely, inhibition of autophagy reduced cilia growth. In atg7-knockout mice, the cilia were shorter in proximal tubule cells. They speculated that mTOR and the proteasome pathways may mediate this reciprocal regulation. Interestingly, cilia may affect the sensitivity of renal tubular cells to apoptosis.

It has been reported that pharmacological agents or genetic manipulations slows or prevents cyst formation and growth by inhibiting apoptosis. Apoptotic or anti-apoptotic BCL2 family members, for example, BIM or BAD, seem to regulate autophagy and could play a role in PKD. Surprisingly, in rats, it has been shown

that apoptosis is more prevalent at early stages of PKD while autophagy seems to be more prominent later in the disease course. However, in ischemic and toxic renal injury models, autophagy seems to precede apoptosis. The level of autophagy in tubular cells lining the cysts is lower than cells in healthy tubules. In Bafilomycin A1 group, the level of the LC3-II was increased in wild type, but not in PKD kidneys. We know that baseline autophagic flux is lower in PKD cells. The mTOR activation could result in the lower level of autophagy in PKD. In fact, MTOR signaling is upregulated in murine models of PKD and in kidneys from patients with PKD. In the studies, the mTOR signaling pathway may modulate disease progression, but, disappointingly, 2 years of treatment with sirolimus had no clear beneficial effect in patients with autosomal dominant PKD. However, the therapy in the treatment group was always stopped because of their side effects.

In the past studies, sirolimus was given at a stage where there were already large cysts in the kidney with clinical CKD. Therefore, scientists guess if starting mTOR inhibition at an earlier stage will prevent cyst formation and CKD, and the achieved drug level might have been too low in the cysts. Others suggested that kidney-specific delivery systems might have more effect on the cyst growth.

Recently, it has been reported that metformin which is a frequently used antidiabetic agent, also affects autophagy and might be beneficial in PKD. Although metformin, 5′ AMP-activated protein kinase (AMPK) activator, increases autophagy and slows cyst formation, scientists have not yet found a cure for PKD. In future, we hope that there will be more and more clinical trials targeting autophagy in PKD and new therapies will improve clinical outcome in these patients.

Inflammation plays a key role in the pathogenesis of glomerulonephritis, but the roles of autophagic regulation of inflammation in glomerular diseases are still not clarified. In glomeruli, it is demonstrated that the role of autophagy was important in podocytes and endothelial cells. Podocytes are terminally differentiated cells, and are poorly regenerative. People found mitophagy was active in embryonic glomeruli, but not in adult ones. This fact may demonstrate that mitophagy plays a critical role in glomerular development, but not in the maintenance of adult glomeruli.

Interestingly, if we block initiation of autophagy by podocyte-specific deficiency of Pik3c3/Vps34, serious proteinuria and earlier onset of glomerular sclerosis and death will be worse. This phenotype is different from the ones seen in autophagy-deficient mice or in liver- and heart-specific Pik3c3/Vsp34-deficient mice. Rather, this kidney phenotype is due to the disruption of the endosomal pathway. The reason for the different phenotypes in tissue-specific Pik3c3/Vps34-deficient mice, autophagic vs nonautophagic ones, awaits to be elucidated (Kimura et al. 2017).

In summary, autophagy largely protects glomeruli, especially unique features of podocytes. While the results in mice with genetic ablations of autophagy-related genes may complicate our understanding of phenotypes. Additionally, we still need to prove the activity status and significance of autophagy in glomerular inflammation. Meanwhile, inactive mitophagy in adult glomeruli may indicate less contribution of mitochondria in glomerular inflammation.

It is reported that autophagy can suppress kidney damage from chronic inflammation. Chronic inflammation, a common condition in CKD patients, affects the kidney.

For example, IL1B can mediate kidney injury though invasion of inflammatory cells, hemodynamic changes, and endothelial dysfunction. Secondary amyloidosis, defined by extracellular deposition of serum amyloid A (SAA) affects most tissues including the kidney. In addition, chronic inflammation indirectly affects the kidney through inflammation-related diseases, such as diabetes and cardiovascular diseases.

Recently, people have started to reveal the molecular mechanisms of how autophagy suppresses NLRP3 inflammasome activation. Mediterranean fever (FMF) is an autosomal recessive disease characterized by episodes of fever with peritonitis, pleural inflammation, arthritis, and systemic amyloidosis, leading to end-stage kidney disease. It is reported that the over activation of the NLRP3 inflammasome and its consequent production of IL1B and SAA/amyloid A can result in FMF.

Because MEFV is an autophagic receptor that recognizes NLRP3, MEFV suppresses inflammasome activation through autophagy. It is reported that once MEFV recognizes NLRP3, autophagic factors such as ULK1 and BECN1/Beclin 1, as well as mammalian Atg8 homologs, are recruited to the MEFV protein complex and activate autophagy. Autophagy in turn mediates specific degradation of NLRP3. It is demonstrated that this MEFV-dependent highly specific type of selective autophagy suppresses NLRP3 inflammasome activity and its consequent amyloid depositions.

35.4 Autophagy and Lupus Nephritis

Systemic lupus erythematosus is one kind of autoimmune diseases which can affect the kidney. It is well known that glomerular deposition of immune complexes is one common feature in lupus nephritis. In a recent study, it is shown that deficiency of LC3-associated phagocytosis causes the presence of autoantibody deposited in glomeruli and systemic inflammation in the mouse kidney, anti-nuclear antibody production in the blood and deposits of immune complexes in the kidney. Thus, scientists speculate that autophagy or LAP-associated proteins may regulate the autoimmune response (Martinez et al. 2016).

In conclusion, autophagy affects systemic inflammatory responses via regulation of the production of cytokines and autoantibodies, or via direct restriction of pathogens. We believe that systemic regulations of autophagy have the potentials to benefit the kidneys, because systemic inflammation has direct toxic effects on the kidney.

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Chapter 36
Autophagy and Diabetic Nephropathy

Jing Cui, Xueyuan Bai, and Xiangmei Chen

Abstract In recent years, diabetic kidney disease has been the main cause of end-stage renal disease; more and more people have faced this serious public health problem worldwide. Autophagy is a conserved multistep pathway that degrades and recycles damaged organelles and macromolecules to maintain intracellular homeostasis. Autophagy plays key roles in several diseases, including kidney diseases. It has been suggested that dysregulated autophagy plays a vital role in both glomerular and tubulointerstitial pathologies in kidneys under diabetic conditions. The advances in our understanding of autophagy in diabetic kidney disease will be helpful for us to discover a new therapeutic target for the prevention and treatment of this life-threatening diabetes complication.

Keywords Advanced glycation end-products · Reactive oxygen species · Rapamycin · Autophagy · Diabetic kidney disease

36.1 The Pathogenesis of Diabetic Kidney Disease

Diabetic kidney disease (DKD) is a serious complication of diabetes mellitus and one of the most significant contributing factors to end-stage renal disease (ESRD). It is reported that around 35–40% of diabetes patients eventually develop DKD, which accounts for a significant increase in mortality in these patients and exacerbates a grave threat to the clinical outcome of diabetic patients. The rapidly increasing prevalence of diabetes and its complications will further increase an already high cost of therapies and economic burden on patients.

The development of DKD in diabetic patients is caused by multifactorial interactions between hemodynamic and metabolic pathways. It is well known that hyperglycemia, hypertension, and genetic pre-disposition are the main risk factors besides

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elevated serum lipids, smoking, overweight or obesity, physical inactivity, and the amount of dietary proteins (Yang et al. 2018).

There are many factors involved in the pathogenesis of DKD, including activation of protein kinase C (PKC), accumulation of advanced glycation end-products (AGEs). In addition, intracellular stress caused by endoplasmic reticulum (ER) stress, oxidative stress, and hypoxia are also the pathological factors in DKD. Meanwhile, hemodynamic changes such as systemic and glomerular hypertension associated with a hyperactive renin-angiotensin system (RAS) are also involved in the progression of DKD. In addition, it has been shown that the metabolic and hemodynamic abnormalities lead to inflammation and kidney fibrosis associated with DKD via the increased production of a variety of cytokines, chemokines, and growth factors.

The prevention and management of DKD have been multi-targeted, advocating a healthy lifestyle and targeting cellular and molecular factors involved in the pathogenesis of this disease. It has been shown that intensive interventions such as glycemic control, blood pressure control, and inhibition of the RAS can delay or decrease the risks for the onset and progression of albuminuria. Unfortunately, these treatments are unable to prevent loss of GFR or progression to ESRD as some patients develop treatment-resistant albuminuria.

Therefore, in order to improve the prognosis of DKD, we need to explore effective therapeutic options. In diabetic kidneys, it has been shown that the level of autophagy is decreased. According to these findings, autophagy deficiency in diabetic kidneys may enhance the susceptibility of kidney cells to diabetes-associated damage, which worse albuminuria and renal function.

In recent years, it has been shown that inflammation is a risk factor for the progression of diabetes nephritis (Najafian et al. 2011). Diabetes is associated with increased intracellular ROS, an activator of the inflammasome. Advanced glycation end-products (AGEs) are irreversibly glycated proteins generated under hyperglycemia and can be endocytosed by kidney proximal tubules for lysosomal degradation, which finally suppress AGEs-induced inflammation. The increased level of AGEs via both overproduction of AGEs and impairment of degradation of AGEs can activate inflammation in diabetic nephropathy. In addition, IL1B (the product of inflammasome activation) pivotally aggravates glucose tolerance. Therefore, inflammasome activation is recognized as a key pathophysiology of diabetic nephropathy.

In lots of studies, it has been indicated that AGEs cause obvious alternations of structure and function in kidney (Ahmed 2005). The formation of AGEs can induce cross-linking of extracellular matrices such as collagen and generalize cellular dysfunction. The level of AGEs production is increased in the hyperglycemic state. Conversely, the clearance of AGEs in the kidneys is decreased, which are responsible for the accumulation of AGEs in DKD patients. It is reported that both the endogenous AGEs in kidneys are degraded in the lysosomes of the proximal tubular epithelial cells.

36.2 The Role of Autophagy in the Development of Diabetic Nephropathy

In DKD, autophagy plays a vital role in maintaining lysosomal homeostasis in podocytes. In addition, it has been reported that AGE overload disrupts lysosomal membrane permeabilization and damages autophagy in DKD.

Considering that the blood glucose levels were comparable, it is possible that AGE accumulation because of autophagy deficiency leads to tubular injury, inflammation, and interstitial fibrosis. In addition, it has been reported that AGEs induce extracellular matrix expansion and epithelial mesenchymal transition and inflammation. After AGE-BSA exposure in the autophagy-deficient PTECs, the level of MCP-1 was increased than in autophagy-competent PTECs. In fact, the mRNA levels of NLRP3, ASC, and caspase-1 and the protein level of IL-1β in the diabetic proximal tubule are increased in vivo. The fact indicates that autophagy plays a vital role in preventing kidney fibrosis and inflammation in DKD by upregulation of lysosomal degradation of AGEs (Takahashi et al. 2017).

Because AGEs can block autophagic flux in cultured PTECs, people speculate that AGEs should accumulate even in autophagy-competent cells treated with AGEs, which has not been the case. In the previous reports, AGEs were directly endocytosed to lysosomes for degradation. In one study, autophagic flux has been measured in vivo by immunostaining for the substrate of autophagy SQSTM1/p62. The results of the above study were the numbers of SQSTM1/p62-positive dots in STZ-treated Atg5F/F:KAP mice compared to vehicle-treated Atg5F/F:KAP mice which were significantly increased, whereas they were comparable between STZ- and vehicle-treated control mice. Therefore, autophagic flux is not completely inhibited in diabetic control mice.

STZ can induce various physiological changes including lipid metabolism besides hyperglycemia which may affect autophagic activity. It is speculated that lysosomal dysfunction and accumulation of AGEs may be result from autophagy deficiency (Chowdhury et al. 2019).

In another streptozotocin-induced diabetes model (type I diabetes model), endothelial-specific autophagy deficiency worsens the diabetic phenotype, which demonstrates that the deficiency of autophagy worsens diabetic nephropathy in vivo. In streptozotocin-induced autophagy-deficient mice, there are severe microalbuminuria, endothelial lesions, and damage in podocytes. High-fat diet challenge induces hyperglycemia with proteinuria, and finally causes damages to podocytes in podocyte-specific autophagy-deficient mice. Autophagy also results in the degradation of AGEs, and thus suppresses inflammation in kidneys (Fig. 36.1).

Currently, the overall activity of autophagy, either activated or inactivated, in diabetic nephropathy is still controversial. It is reported that hyperinsulinemia due to hyperglycemia may suppress autophagic activity through MTOR activation. Importantly, this suppressive function of insulin seems to have a tissue-specificity, i.e., insulin suppresses autophagy in muscles whereas amino acids suppress autophagy in liver.

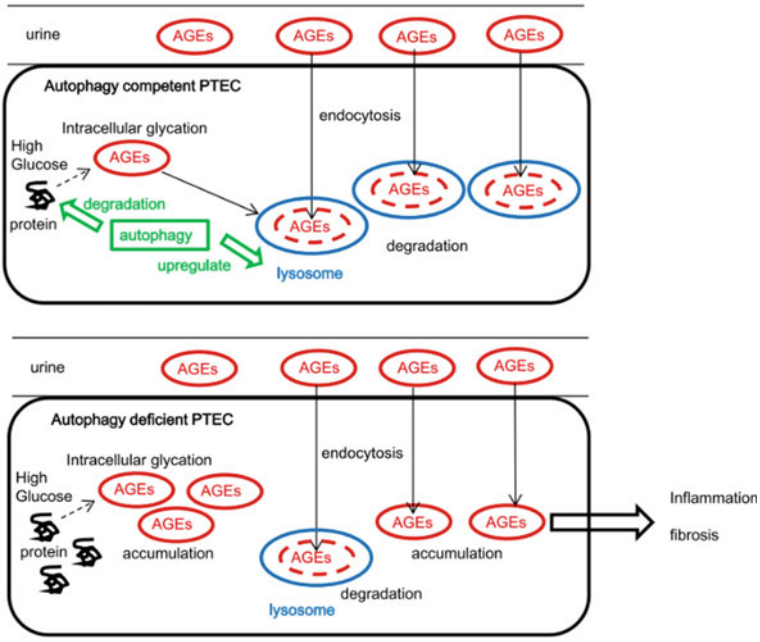

Fig. 36.1 It is illustrated that possible mechanisms by which autophagy protects PTECs from the accumulation of AGEs under diabetic conditions. Autophagy inhibits the accumulation of endogenous AGEs by degrading intracellular proteins and exogenous AGEs by upregulating lysosomal functions, resulting in inhibiting inflammation and fibrosis in DKD (Takahashi et al. 2017)

Conversely, it is also suggested that autophagy can be induced under hyperglycemia due to the production of ROS or direct cytotoxicity of hyperglycemia. In future, we still need to be determined whether over-nutrition status and hyperinsulinemia could suppress autophagy in kidney.

Moreover, what further modifies the autophagic activity under diabetic condition is the presence of diabetic byproducts and resulting complications. AGEs suppress autophagic/lysosomal degradation activity. Similar phenomena were reported under hyperlipidemia (high-fat diet challenge), a common metabolic complication seen in diabetes. Mechanistically, it is suggested that AGEs impair lysosomal membrane permeability and function. Conversely, autophagy upregulates lysosomal biogenesis and function via nuclear translocation of TFEB (transcription factor EB), a key regulator of lysosomal biogenesis.

In autophagy-deficient kidney tubular cells, it is shown that neither TFEB nuclear translocation nor lysosomal biogenesis upon AGEs challenge. The facts suggest a critical role of the autophagy-TFEB axis against AGEs. The scientists speculate that these byproducts and complications of diabetes may be associated with autophagic regulation of inflammation of the kidney in diabetes.

In conclusion, autophagy indeed plays a protective role in diabetic nephropathy. However, it is not clear whether autophagy is active or inactive in DKD. It is acknowledged that inflammation is the vital factor in the DKD pathogenesis. Importantly, autophagy may play a protective role against kidney inflammation in DKD, which may help us to explore the mechanism of the DKD.

It is the fact that in diabetic nephropathy models, the activation of mTOR in the proximal and distal tubules and the downregulation of the AMP-activated protein kinase pathway result in autophagy inhibition. In addition, the scientists also found that prolonged exposure to high glucose levels induced apoptosis and autophagy in tubular epithelial cells in vitro. Meanwhile, in the diabetic nephropathy, elevated ROS production and ER stress both can upregulate autophagy. In addition, autophagy is also induced by lipotoxicity in tubular cells via modulating fatty acid β-oxidation.

36.3 Interventional Treatment of Diabetic Nephropathy by Regulating Autophagy Activity

It is reported that a phytochemical ferulic acid protects against varied diseased conditions. In one study, the scientists explored the ameliorative role and mechanisms of ferulic acid in averting STZ-mediated nephrotoxicity. In vivo, they administered a single intraperitoneal injection of streptozotocin (50 mg/kg) in experimental rats to induce diabetes. In diabetic rats, the results were the increased level of blood glucose, as well as kidney to body weight ratio, a decrease in serum insulin level, severe kidney tissue damage and dysfunction. The participation of oxidative stress in hyperglycemia-triggered renal injury was demonstrated by elevation of intracellular ROS level, altered mitochondrial membrane potential, and cellular redox balance impairment. In the kidneys of ferulic acid group, kidney damage, apoptosis, inflammation, and defective autophagy were markedly ameliorated, probably via the underlying mechanism for such protection, such as AGEs, MAPKs, NF-κB-mediated inflammatory pathways, autophagy induction, and so on. In vitro, ferulic acid could decrease excessive ROS generation, active autophagy, and prevent apoptotic death of cells under high glucose environment. When autophagy is inhibited, the protective effect of ferulic acid is significantly eradicated in high glucose-mediated cell death. In conclusion, it has been confirmed that ferulic acid play antioxidant, anti-inflammatory, anti-apoptotic activities and role in autophagy, which could avoid oxidative stress-mediated renal cell damage (Chowdhury et al. 2019).

In another study, the role of spironolactone in podocyte loss and autophagy has been investigated. It is reported that spironolactone decreased the urinary albumin excretion, fasting glucose levels and lipids, and alleviated kidney damage. Spironolactone also increased the expression of the podocyte-specific markers WT1 and NPHS2, as well as the autophagic markers Beclin1 and LC3B. In addition, spironolactone not entirely blocked the renin–angiotensin–aldosterone system (RAAS) by

regulating the ACE1, ACE2, and aldosterone levels. In one word, spironolactone promoted autophagy in podocytes and further alleviated DN through partially impeding the RAAS (Dong et al. 2019).

It is the fact that podocyte loss and apoptosis play a crucial role in the progression of DKD. One widely used Chinese herb called tripterygium glycoside (TG) has exerted comprehensive protective effects on preventing DKD progression. In a new study, people assessed the podocyte protective effect of tripterygium glycoside on DKD by the potential role of activation of autophagy and downregulating β-arrestin-1. In vitro, tripterygium glycoside and small interfering RNA (siRNA) of β-arrestin-1 were added to 10% db/db mice high-glucose serum to induce podocytes. In DKD mouse serum treated podocytes, pretreatment of tripterygium glycoside significantly ameliorated podocyte apoptosis, increased nephrin and podocin levels and inhibited expression of β-arrestin-1. Meanwhile, levels of autophagic activity were significantly higher. They demonstrated that silencing β-arrestin-1 upregulated autophagic activity and ameliorated podocyte apoptosis. In addition, silencing β-arrestin-1 in combination with tripterygium glycoside enhanced the levels of LC3-II and LC3-II/LC3-I ratios and reduced the expression of p62. Finally, in podocyte apoptotic rate was reduced notably in DKD serum + siRNA-β-arrestin-1 + TG group compared to DKD serum + siRNA-β-arrestin-1 group, and the levels of nephrin and podocin were increased compared to treatment with siRNA-β-arrestin-1 only. They demonstrated that tripterygium glycoside provided protection against podocyte injury induced by high-glucose serum, and that this effect was mediated by the concomitant activation of autophagy and downregulation of β-arrestin-1 (Zhan et al. 2019).

Generally, autophagy activity is inhibited under diabetic conditions, thus new therapeutic strategy has been focusing on restoring autophagy. In clinic, diet or calorie restriction is an important glycemic control therapy in diabetic patients and plays a renoprotective role in several metabolic or age-related kidney diseases. It is essential that activation of autophagy plays an indispensable role in calorie restriction-mediated anti-aging effects. Therefore, we believe that a calorie restriction regimen can activate autophagy which should be a potent therapeutic strategy to prevent DKD. In fact, researchers demonstrated that calorie restriction can restore autophagy activity in PTECs and attenuate renal damage in type 2 diabetic Wistar fatty rats. However, in recent human clinical studies, the quality of diet affects the development of insulin resistance and new onset of diabetes. In addition, under diabetic conditions, it is reported that a high energy/nutrient intake along with a low-protein diet may cause an insufficiency of diet restriction. Finally, we also need to identify the types of carbohydrates, amino acids, and fatty acids in different diet regimens that can result in a better prognosis.

In addition, it is the fact that drugs that can activate autophagy which affect mTORC1, AMPK, and SIRT1 may have therapeutic potency in DKD treatment. Rapamycin can induce autophagy and improve glomerular and tubular damage in DKD. However, it has been well-recognized that the side effects of long-term or complete mTORC1 inhibition.

Currently, it is still under debate whether mTOR inhibition by rapamycin or other mTOR inhibitors is safe and effective for treating DKD. In addition, it is

also being explored that targeting AMPK and SIRT1 with activators such as resveratrol, metformin, and AICAR. It has been demonstrated that the above agents activate autophagy in different animal models of diabetes and protect the kidney from diabetic injury, making them be attractive therapeutic options for DKD. Moreover, the use of antioxidants to counter oxidative stress or chemical chaperons to reduce ER stress and to restore autophagy activity may also be a hopeful therapeutic approach to treat the diabetic kidney disease.

36.4 Conclusion

It is no doubt that autophagy would be a key therapeutic option in kidney diseases. However, modulation of autophagy in cancer therapy can worsen kidney function, which is demonstrated that general modulation of autophagy might result in unexpected side effects on the kidney. It is the fact that rough and bulk modulation of autophagy, therefore, may result in unwanted side effects in the kidney.

We still need to explore in future what the targets of autophagy are and how autophagy recognizes these specific targets in kidney diseases. For example, the mechanism and the proteins that are degraded in kidney diseases by autophagy are scarcely known. It is obvious that mitochondria are the targets of autophagy in kidney. However, how autophagy selectively recognizes damaged or depolarized mitochondria in unhealthy ones awaits determination. So far, it has not been identified as receptors of mitophagy for kidney diseases. Of course, specific targeting of autophagic targets would promote precision medicine. In other words, precise degradation of autophagic targets by precision autophagy would benefit kidney disease patients.

It has been reported that autophagy has dual roles (both activating and suppressing) in the kidney diseases, so the precise mechanistic study of these regulatory processes would also benefit kidney disease patients. Because autophagy is a metabolic process, one potential regulatory mechanism may be via metabolism. The modulations of autophagy may have a vital role in immunometabolic regulations in kidney diseases.

In conclusion, autophagic regulation protects most kidney diseases. In future, we need to clear the precise role of autophagy in kidney diseases.

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Part VIII
Autophagy, Hepatology and Gastroenterology

Autophagy, or cellular self-digestion, is a cellular pathway crucial for development, differentiation, survival, and homeostasis. Its implication in human diseases has been highlighted during the past decade. Recent data show that autophagy is involved in major fields of hepatology and gastroenterology. Autophagy provides starved cells with amino acids, glucose, and free fatty acids for use in energy production and synthesis of new macromolecules, and also controls the quality and quantity of organelles such as mitochondria. Although the efforts of early investigators contributed markedly to our current knowledge of autophagy, the identification of autophagy-related molecules and pathways represented a revolutionary breakthrough in our understanding of the physiological roles of autophagy in the liver and gastrointestinal system. A growing body of evidence has shown that autophagy contributes to the basic functions of the liver and gastrointestinal system. In this chapter, we describe the roles of autophagy in hepatic and gastrointestinal function under healthy and disease conditions.

Chapter 37
Autophagy and Liver Diseases

Jia Fan, Yinghong Shi, and Yuanfei Peng

Abstract Autophagy plays an important role in the physiology and pathology of the liver. It is involved in the development of many liver diseases such as α-1-antitrypsin deficiency, chronic hepatitis virus infection, alcoholic liver disease, nonalcoholic fatty liver disease, and liver cancer. Autophagy has thus become a new target for the treatment of liver diseases. How to treat liver diseases by regulating autophagy has been a hot topic.

Keywords Autophagy · Liver disease · Cancer

It has been demonstrated that autophagy plays an important role in the pathophysiology of the liver (Shi et al. 2011a, 2011; Ueno and Komatsu 2017; Di Fazio and Matrood 2018). It contributes to the development of many liver diseases, including α-1-antitrypsin deficiency, chronic hepatitis virus infection, alcoholic liver disease, nonalcoholic fatty liver disease, and liver cancer (Ueno and Komatsu 2017; Di Fazio and Matrood 2018).

37.1 Autophagy and α-1 Antitrypsin Deficiency

α-1-antitrypsin (α1-AT) is a serum glycoprotein synthesized in the liver. α-1-antitrypsin deficiency (ATD) is a congenital metabolic disease caused by a deficiency in the anti-protease component of the blood (α1-AT). ATD is an autosomal dominant disease caused by Z-point mutation of the AT gene. Most patients with α1-AT deficiency are homozygous mutations (called ZZ or "PiZZ"). The mutant protein is called α1-antitrypsin Z mutant (ATZ), which is prone to fold and has a tendency to aggregate. Aggregated ATZ accumulates in the endoplasmic reticulum of hepatocytes, which causes endoplasmic reticulum stress and cell death (Perlmutter 2006). Hepatocyte death causes compensatory hepatocyte proliferation. Periodic liver cell damage, death, and compensatory hyperplasia, leading to liver

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disease, from mildly elevated enzymes to liver fibrosis, cirrhosis, and hepatocellular carcinoma (Teckman and Mangalat 2014). Decreased AT in serum leads to destruction of lung tissue, proteolytic damage of the connective tissue matrix of the lung, and subsequent emphysema (Janssen et al. 2019; Perlmutter 2016). The classical form of α1-antitrypsin deficiency (ATD) has two types of main manifestation, intrahepatic (liver fibrosis, cirrhosis, and hepatocellular carcinoma) and extrahepatic (chronic obstructive pulmonary disease). A typical α1-antitrypsin deficiency is a serious childhood liver disease. Clinically, it often leads to neonatal hepatitis, liver cirrhosis, liver cancer, and emphysema in infants and adults. Clinically, only 8–10% of ATD forms a clinically significant liver disease (Sveger and Eriksson 1995). This difference in liver disease phenotype suggests a genetic modification or protective response which is responsible for the treatment of mutant ATZ in the endoplasmic reticulum. Early studies using yeast and mammalian cell lines indicated that the proteasome pathway is involved in the intracellular degradation of mutant ATZ. However, the proteasome pathway does not completely treat ATZ. Studies after the establishment of the human ATD cell model (genetic engineering overexpression ATZ) indicate that autophagy is the main pathway for ATZ molecular degradation (Chu et al. 2014). The accumulation of polymerized ATZ molecules in the cells induces autophagy, and ATZ is introduced into autophagic vacuoles. The accumulation of ATZ molecular significantly increased in the autophagy defect system. In PiZ mice, increased liver damage after ATZ load increased, and drug-induced autophagy reduced liver damage (Hidvegi et al. 2010; Kaushal et al. 2010). In the transgenic ATD mouse model constitutively expressing ATZ in the liver, the basal level of autophagosomes accounted for 2.5% of hepatocyte cytoplasm, while that accounted for only 0.5% in the control C57/BL6 mice, indicating autophagy enhancement in the liver of ATZ mice (Teckman et al. 2002). ATZ autophagosomes are widely present in hepatocytes in AT-deficient mice and AT-deficient patients. The use of the autophagy inhibitor 3-methyladenine (3-MA), wortmannin and LY-294002 resulted in reduced ATZ degradation. In addition, the ATZ-degrading yeast model also found that aggregated ATZ is degraded by the autophagy pathway (Kruse et al. 2006a). This was further confirmed by ATG5 knockdown human cell ATD model that simultaneously overexpresses ATZ. ATZ degradation is impaired and ATZ accumulates in large amounts after autophagy inhibition. Through the GFP-LC3 transgenic mouse animal model and liver-specific induction of ATZ, it was found that ATZ accumulation in hepatocytes is sufficient to activate autophagy. The molecular mechanism of ATZ is not well understood. Genome-wide analysis using mammalian cells and mouse ATD models has shown that accumulation of ATZ cells is associated with NF-κB and TGF-β signaling pathways (Chu et al. 2014). Insulin metabolic pathways are also involved. Wild-type or mutant AT model study using transgenic technology and Caenorhabditis elegans model showed a significant decrease in ATZ accumulation in insulin-deficient patients (Hidvegi et al. 2015).

The treatment option of ATD is limited. Usually, liver transplantation is the only curative treatment for ATD. Purified AT replacement therapy is not satisfied. The clinical efficacy is not significant in the patients with ATD-related COPD and the

results are puzzling (Dickens and Lomas 2011). In recent years, autophagy has provided new hope for the treatment of ATD. Using autophagy enhancer to induce autophagy treatment of insoluble ATZ polymer and reduce its cell load has become a new therapeutic strategy (Chu et al. 2014). A number of drugs have been studied for the treatment of ATD (Chu et al. 2014). Rapamycin and carbamazepine (CBZ) can significantly reduce intrahepatic ATZ and reduce liver fibrosis and other liver damage parameters (Wang and Perlmutter 2014; Teckman and Mangalat 2014). Among them, CBZ is the most promising. Both cell ATD model and PiZ mouse ATD animal model studies have confirmed that CBZ can mediate significant degradation of ATZ, reduce liver ATZ load, and mitigate liver fibrosis (Hidvegi et al. 2010; Teckman and Mangalat 2014). Clinical trials of carbamazepine in end-stage liver disease are currently underway (ClinicalTrials.gov. Available from: http://clinicaltrials.gov/show/NCT01379469) and the results will be announced in the near future. In addition, the ATD nematode model was used for high-throughput drugs screening. A total of 1280 drugs were screened and five drugs were identified that can reduce ATZ load in a dose-dependent manner. Interestingly, four of them have the effect of enhancing autophagy. Two of the compounds (fluphenazine and pimozide) belong to the phenothiazine family. They are structurally related to tricyclic antidepressants and are a family member of carbamazepine. Further validation in mammalian cells and mouse ATD models confirmed the effect of fluphenazine (Sinha et al. 2014). Fluphenazine enhances autophagy and reverses the phenotypic effect of ATZ accumulation in nematodes and reduces the ATZ load in mammalian cell line cell models. In the PiZmouse model, fluphenazine reduces liver ATZ accumulation and reduces liver fibrosis. These results indicate that fluphenazine can reduce the toxicity of ATZ protein aggregation in vivo. As it has been safely used in humans it can be rapidly converted to clinical trials of ATD liver disease. Several other drugs have not been validated, but they have been verified in Huntington's disease for degrading aggregation-prone proteins. Other autophagy inducer agents such as glucosamine and N-acetylglucosamine can enhance autophagy in mammalian cells through non-mTOR dependent pathways. Another hypolipidemic drug, ezetimibe (Ezetimibe) can also induce autophagy in hepatocytes and small intestinal epithelial cells. Ezetimibe can reduce ATZ accumulation in primary cultured hepatocytes (genetically engineered hepatocyte model for genetic engineering of ATZ) (Yamamura et al. 2014; Garcia-Calvo et al. 2005). Ezetimibe can significantly reduce ATZ load and mutant ATZ deposition to improve liver damage caused by ATD by inhibiting NPC1L1 to activate autophagy (Yamamura et al. 2014). Tat-Beclin 1 polypeptide is a potent inducer of autophagy which enhances the degradation of mutant Huntingtin and is also a potential drug candidate. Studies have also shown that ursodeoxycholic acid derivatives (norUDCA) can promote ATZ degradation and alleviate disease by inducing autophagy through AMPK/ULK1 pathway (Tang et al. 2018). Recent studies have also shown that gene therapy can alleviate the protein degradation toxicity of ATZ. TFEB is a major gene that regulates autophagy and lysosomal gene expression. Studies have shown that TFEB induces autophagy-dependent ATZ clearance in the ATD mammalian cell model. In the PiZ mouse model, adenovirus-mediated TFEB transduction can significantly promote ATZ autophagy degradation and reduce

liver fibrosis in vivo, indicating that gene therapy may be one of the methods to treat ATD liver disease (Pastore et al. 2013). Subsequent studies have shown that in addition to intrahepatic effects, the transfer of the transcription factor TFEB upregulates autophagy, which also attenuates the extrahepatic effects of ATZ and reduces pulmonary fibrosis emphysema (Hidvegi et al. 2015).

37.2 Autophagy and Liver Neoplasm

Autophagy plays an important role in tumorigenesis and tumor progression (Yazdani et al. 2019). However, the role of autophagy in hepatocarcinogenesis is reported to be paradoxical role in (Yazdani et al. 2019). Collectively, autophagy appear to be tumor-suppressive at the early stage of hepatocarcinogenesis and contributes to tumor progression and treatment resistance.

The tumor-suppressive function of autophagy was primarily established on the gene knockout mice. Genetic evidence shows that mouse with essential autophagy genes knockout can form hepatic neoplasm. Several gene has been identified as tumor suppressor, including BECN1 (Beclin 1), ATG5, and ATG7. Murine gene knockout model demonstrates that systematic deletion of ATG5 will form hepatoma (Takamura et al. 2011). And more interesting is that ATG deletion can induce various benign hepatic tumors and the tumors only in liver (Takamura et al. 2011). It can be observed in liver-specific ATG7 gene deletion in mouse model (Takamura et al. 2011). It has been shown that monoallelic deletion of the autophagic gene Beclin 1 promotes tumorigenesis. Heterozygous disruption of Beclin 1 reduces autophagy and increases the frequency of spontaneous malignancies including liver cancer. However, homozygous Beclin 1 knockout mice cause embryonic lethality. Thus, Beclin 1 has been demonstrated as a haploinsufficient tumor suppressor gene (Liang et al. 1999; Yue et al. 2003; Qu et al. 2003). In addition to increasing the frequency of spontaneous tumors, Beclin $1^{+/-}$ mutations also accelerate the progression of precancerous lesions caused by hepatitis B virus and increase cell proliferation in vivo (Qu et al. 2003) Recent studies have also shown that Beclin 1 and Bax inhibit HCC proliferation, invasion, metastasis, and angiogenesis (Qiu et al. 2014). These data from animal models provide clear evidence that autophagy is an important tumor suppressor that inhibit tumor formation. P62 is a selective autophagy substrate. Autophagy defects cause continuous p62 expression, accumulate, and promoting tumor progression (Mathew et al. 2009). P62 aggregation is often detected in human cancer including liver cancer (Inami et al. 2011). Human hepatocellular carcinoma is associated with p62 accumulation in MDBs. Beclin 1 gene heterozygous mutation is associated with p62 aggregation in hepatic tumor (Yue et al. 2003; Qu et al. 2003; Komatsu et al. 2007; Mathew et al. 2009). Liver-specific ATG7 knockdown forms hepatocellular adenomas in mice with p62 accumulation and subsequent Nrf2 activation, whereas sustained activation of Nrf2 by p62 leads to human liver cancer formation (Inami et al. 2011). Tumor size was significantly reduced in liver-specific ATG7 knockout and P62 knockout mice (Takamura et al. 2011). In addition, the

tumor suppressor gene p53 has a dual regulation of autophagy. In the nucleus, p53 can act as an autophagy inducing transcription factor. In the cytoplasm, p53 acts as an inhibitor of autophagy, and its degradation is required to induce autophagy in cells (Levine and Abrams 2008). Another evidence for the inhibition of autophagy on HCC is that some studies have demonstrated autophagy defects in HCC tissue cells. By detecting p62 in HCC tissues, paracancerous tissues, and HCC cell lines, it was demonstrated that HCC has autophagy defects. And autophagy is less active in HCC cancer tissues than in adjacent tissues (Bao et al. 2014). Autophagy levels are inversely related to the degree of malignancy of HCC. The basal expression of autophagy genes and the corresponding autophagy activity under starvation conditions are different in liver cancer cell lines with different malignant phenotypes. It seems that autophagy defects are associated with higher HCC malignant phenotypes (Ding et al. 2008). The inhibition of autophagy on liver tumors is also related to the inhibition of inflammation by autophagy. Inflammation is an important factor in the development of liver cancer. The occurrence and development of HCC is highly correlated with persistent inflammatory stimulation. Inhibition of autophagy can lead to a continuous increase in inflammation (Bujak et al. 2015; Zhong et al. 2016). LC3B knockout mice are more susceptible to endotoxin-induced LPS death (Nakahira et al. 2011). Studies show that autophagy can prevent cancer progression by inhibiting inflammation (Zhang et al. 2014a; Shibutani et al. 2015; Saitoh et al. 2008). In the early stage of liver cancer, silencing of Atg5 gene causes loss of autophagy and promotes the production of inflammatory and fibroblast factors in Kupffer cells, enhanced NF-κB signaling pathway and increased IL-1α/β expression, promoting mitochondrial ROS-mediated inflammation and fibrosis, and ultimately promotes tumorigenesis and progression, while blocking mitochondrial ROS or IL-1 receptors prevents fibrosis, inflammation, and tumor formation caused by Atg5 gene knockout (Sun et al. 2017). Some tumor-inhibiting molecules are associated with autophagy induction. These protein molecules are usually abnormally expressed in liver tumors and result in attenuated autophagy inhibition. For example, DDX5, a tumor suppressor protein in the liver, binds to p62 and interferes with P62/TRAF6 interaction to induce autophagy and inhibit tumors. Its expression is significantly low in human liver cancer tissues (Zhang et al. 2019). The chemokine CXCL17 is highly expressed in human HCC tissues. Silencing CXCL17 induces autophagy and results in reduced tumor proliferation (Wang et al. 2019). miR-7 and miR-85 are recognized as tumor suppressor microRNAs. miR-7 in HCC cells increases autophagy activity by targeting the mTOR pathway and results in decreased cancer cell proliferation. The miR-7 levels are significantly down-regulated in HCC (Wang et al. 2017). miR-85 transfected human HCC cells HepG2 upregulates autophagy activity and leads to cell cycle arrest. The miR-375 is down-regulated in HCC cell lines and tissues (Zhou et al. 2017). miR-375 can down-regulate ATG7 and inhibit the conversion of LC3I to LC3II to inhibit autophagy. In vivo studies show that miR-375 overexpression can significantly inhibit tumor growth (Chang et al. 2012). These results indicate that autophagy plays an inhibitory role in the development of liver tumors, and autophagy defects may promote tumor progression (Shi et al. 2009). The therapeutic value of autophagy to inhibit the occurrence of liver tumors is that

autophagy induction can be used to prevent liver cancer. Precancerous lesions can utilize autophagy to inhibit inflammation, tissue damage, genomic instability, etc., and autophagy may be beneficial in preventing liver cancer. For example, in patients with α-1 antitrypsin deficiency which can lead to liver cancer, induction of autophagy in hepatocytes can inhibit hepatic inflammation and carcinogenesis (Perlmutter 2009). Carbamazepine (CBZ) has been shown to induce increased autophagy, reduced α-1 antitrypsin loading, and reduced liver fibrosis and cancer risk in ATD mouse model, suggesting that enhancing autophagy may be an effective way to prevent liver cancer (Hidvegi et al. 2010).

Autophagy also promotes liver cancer progression. There is increasing evidence that autophagy is required for tumor cells, including liver cancer cells, to survive in a variety of adverse environments such as starvation, growth factor deficiency, hypoxia, injury stimuli, and drug therapy. Hypoxia-induced oxidative stress is one of the most prominent features of all solid tumors. Autophagosomes are common in the hypoxic region of the tumor, indicating an increase in autophagy levels. Inhibition of autophagy by Beclin 1 knockdown leads to tumor cell death (Degenhardt et al. 2006). Cells overcome hypoxic stress by regulating their metabolism and energy requirements under hypoxic conditions. Autophagy is one of the important mechanisms. Hypoxia-induced autophagy in HCC cells is dependent on hypoxia-inducible factor (HIF1). HIF1-upregulates BNIP3 and BNIP3L proteins that bind to BCL-2 protein inhibits the destructive interaction between BCL-2 and Beclin 1 and induces autophagy. In addition to HIF1, hypoxia also upregulates Egr-1 (early growth response gene-1) expression, which regulates autophagy in HCC cell lines to promote migration (Peng et al. 2016). Autophagy can control excess ROS production under hypoxic stress by removing damaged organelles from the cells. Impaired and non-functional mitochondria are the major sources of cellular ROS production. Induction of mitochondrial autophagy helps to clear these damaged mitochondria to maintain cellular function and bioenergy (Liu et al. 2017). Generally, increased autophagy in basal cells is required for the continued growth of tumor cells. In this circumstance, autophagy provides a key intermediate for the metabolism required for tumor cell growth. RAS-driven tumor formation leads to elevated levels of basal autophagy in cells. In vivo ATG5 or ATG7 knockdown can inhibit RAS-driven tumor formation (Guo et al. 2011). Autophagy also regulates inflammatory immune responses by releasing and degrading damage-related molecules to promote cell survival and growth, including HMGB1, histones, ATP, mitochondrial DNA, and mitochondrial transcription factor A (Chen et al. 2015; Huang et al. 2014; Liu et al. 2015). Cancer stem cells have high tumorigenicity, metastasis ability, and drug resistance. They play an important role in the occurrence and development of liver cancer (Li and Zhu 2019). Autophagy plays a role in regulating the adaptation of liver cancer stem cells to hypoxic and hypotrophic tumor microenvironments (Song et al. 2013; Li et al. 2016a). Song et al. (2013) showed that autophagy contributes to the survival of CD133-positive liver cancer stem cells in hypoxic and low-trophic tumor microenvironment. Under hypoxic and low nutrient conditions, the proportion of CD133-positive hepatoma cells increased and the stem cells of liver cancer cells were more prominent in stem property. When the level of autophagy decreased after treatment

of the autophagy inhibitor chloroquine, the number of CD133-positive hepatoma stem cells increased significantly and the ability of self-renewal is weakened. Recent studies have also shown that autophagy plays an important role in liver cancer metastasis. There is increasing evidence of an upregulation of autophagy during tumor metastasis (Debnath 2008; Peng et al. 2013c). Autophagy plays an important role in cell anoikis tolerance and cell colonization of distant metastases. Inhibition of autophagy can reduce liver metastasis of liver cancer. Its mechanism is related to promoting liver cancer metastasis cells to resist anoikis and promote colonization (Peng et al. 2013a, c). Epithelial-mesenchymal transition (EMT) cells acquire the ability to invade and migrate, producing interstitial properties. Autophagy promotes EMT through the TGF-β/Smad3 pathway to promote metastasis (Li et al. 2013). However, it is still controversial as there are studies showing that autophagy can also inhibit EMT. The effect of autophagy on cell migration and invasion seems to be minimal (Peng et al. 2013a, c), although several studies have shown that induction of autophagy in starved cells upregulates the expression of matrix metalloproteinase-9 (MMP9) (Li et al. 2013).

Autophagy can also affect the occurrence and development of tumors by regulating the pathogenic factors of liver cancer. But the effects are different depending on the pathogenic factors. HBV infection is the most important cause of liver cancer. HBV virus can regulate autophagy to enhance self-replication, and subsequently cause hepatocyte necrosis, inflammation, regeneration, and ultimately lead to liver cancer (Sir et al. 2010). In liver cancer cells and mice, autophagy is activated during HBV infection, and enhanced autophagy promotes HBV replication (Li et al. 2011; Tian et al. 2011). Hepatitis C virus is closely related to liver cancer. Hepatitis C virus can also use autophagy for replication to promote liver cancer. Hepatitis C virus RNA replication can induce UPR and CHOP expression, thereby activating autophagy by promoting autophagosome formation (Sir et al. 2008; Ait-Goughoulte et al. 2008). Some ATGs, including Beclin 1, ATG4b, ATG5, and ATG12, are involved in the translation of viral mRNA and replication initiation (Dreux et al. 2009). RNA replication can block the maturation of autophagosomes and promotes their activity by utilizing autophagosome content (Sir et al. 2008). In addition, HCV can prevent itself from being identified and degraded by autophagy mechanisms (Alavian et al. 2011; Rautou et al. 2010). Chronic drinking has been recognized as promoting liver cirrhosis and liver cancer. A possible mechanism is that alcohol causes liver damage and promotes liver cancer by inducing oxidative stress. The ability of autophagy to regulate hepatic steatosis and oxidative stress cell death suggests an important role for autophagy in ethanol-induced liver disease, including liver cancer (Czaja 2011; Wang et al. 2010b). However, the exact role of autophagy in alcohol-related liver cancer remains to be clarified. Nonalcoholic fatty liver disease is a common chronic liver disease that increases the risk of fibrosis and liver cancer. Autophagy promotes lipid droplet degradation, called lipophagy (Singh et al. 2009). In hepatic stellate cells, inhibition of autophagy promotes accumulation of lipid droplets and reduces fibrosis which may eventually progress to cirrhosis and liver cancer, indicating that autophagy may prevent the formation of NAFLD-associated liver tumors (Hernandez-Gea et al. 2012).

In recent years, autophagy has been found to be associated with treatment tolerance in liver cancer. Inhibition of autophagy has become a potential strategy for the treatment of liver cancer. For example, hypoxia can enhance the chemoresistance of hepatocarcinoma cells by inducing autophagy. Inhibition of autophagy can restore the sensitivity of hepatoma cells to chemotherapy, indicating that autophagy plays a role in promoting cell survival in cell death induction by chemotherapy drugs (Song et al. 2009). Many current cancer treatments (such as inhibition of angiogenesis and growth factor receptors) and some conventional treatments (radiation and chemotherapy) cause metabolic stress and induce autophagy. Autophagy inhibitors can be used in combination with these therapies to eliminate the protection of autophagy and improve efficacy (Wu et al. 2012). The antimalarial drugs chloroquine and hydroxychloroquine are currently being evaluated in preclinical studies and clinical trials, while small molecule specific autophagy inhibitors are being developed.

It should be noted that most of the current research is based on hepatocellular carcinoma. It is reported that autophagy plays a role in other two types of liver cancer (cholangiocarcinoma and hepatoblastoma). For example, autophagy is activated under starvation conditions or after chemotherapy in cholangiocarcinoma cells and hepatoblastoma tissues in xenograft model. Inhibition of autophagy with autophagy inhibitors and small interfering RNA can significantly increase apoptosis in cholangiocarcinoma cells and sensitized chemotherapeutic drug-induced cell death (Hou et al. 2011). In addition, inhibition of autophagy by regulation of autophagy genes can inhibit hepatoblastoma formation (Hou et al. 2011; Chang et al. 2011). However, the exact role of autophagy in cholangiocarcinoma/hepatoblastoma and mechanism underlying remain to be further studied.

The important role of autophagy in liver cancer has made it a new therapeutic target or a new treatment strategy for liver cancer. There is currently no consensus on the use of autophagy drugs for the treatment of cancer. Theoretically, autophagy inhibitors are used to eliminate or attenuate the autophagy-induced protection of chemotherapy or other treatments to increase tumor cell killing and enhance the therapeutic effect. The autophagy inducer is used to induce autophagic death to enhance tumor cell killing of chemotherapy. Autophagy inhibitors are generally considered to have synergistic effects with chemotherapy or radiotherapy in the process of killing cancer cells. For example, sorafenib and bortezomib can induce autophagy through the PI3K-Akt pathway and the Akt pathway in the treatment of liver cancer. The combination of autophagy inhibitors 3-MA or CQ can enhance their cytotoxic effects (Shimizu et al. 2012; Hui et al. 2012; Yu et al. 2013a). Cisplatin or oxaliplatin has similar results (Xu et al. 2012; Ding et al. 2011). But some other drugs are not. The anticancer drug nilotinib (Yu et al. 2013b), cannabinoids tetrahydrocannabinol, JWH-015 (Vara et al. 2011) or the histone deacetylase inhibitor SAHA (Liu et al. 2010) can induce autophagy by the AMPK signaling pathway. The combined use of autophagy inhibitors reduces but rather enhances the efficacy of liver cancer. These results show the complexity of autophagy for treatment of liver cancer. Due to the multi-faceted nature of autophagy in liver cancer, applications should be determined according to the specific conditions (Amaravadi et al. 2007, 2011). Autophagy is also a mechanism of cell death, which promotes autophagy-dependent cell death by promoting

apoptosis or autophagic cell death (Eisenberg-Lerner et al. 2009; Shen and Codogno 2011; Edinger and Thompson 2004). For example, in tumor cells with defective apoptotic mechanisms, induction of autophagic cell death is an effective method to promote cell death (Maycotte and Thorburn 2011). Table 37.1 summarizes autophagy inducing agents and inhibitors that have been developed in vitro and in vivo systems as well as in clinical trials. I. Autophagy inducer: (1) Rapamycin and its analogs: the key signal cascade of autophagy in hepatoma cells is PI3K/Akt/mTOR pathway which regulates cell growth, proliferation, angiogenesis, and apoptosis (Sabatini 2006). This pathway is activated at 15–41% HCC and mTOR inhibitors have anti-HCC activity (Sieghart et al. 2007). Rapamycin (sirolimus) has been widely used as an autophagy inducing agent and drug for anti-proliferation and anti-angiogenesis. Rapamycin and its derivatives such as everolimus (RAD001) have been shown to have antitumor activity in preclinical studies of liver cancer (Huynh et al. 2009); (2) tyrosine kinase inhibitors: tyrosine kinases in tumor progression play a major role. It has been developed for cancer treatment. Sorafenib in combination with the HDAC inhibitor SAHA enhances liver cancer death by inducing autophagy (Martin et al. 2009; Park et al. 2008). However, doxorubicin (DOX)-induced autophagic cell death can be inhibited by sorafenib, which promotes cell cycle progression, improves survival, and reduces autophagy in liver cancer cells (Manov et al. 2011). Therefore, the possible antagonism of sorafenib in combination with doxorubicin needs further consideration; and (3) Other: NPC-16, a new naphthalimide-like polyamine conjugate, can induce autophagy and apoptosis in liver cancer cells. The mTOR signaling pathway has been shown to be involved in NPC-16-mediated autophagy (Xie et al. 2011). Berberine can also induce autophagic cell death in hepatocellular carcinoma cells. It inhibits the mTOR pathway by inhibiting Akt activity through upregulating the AMPK signal transduction pathway to induce autophagic death of hepatocellular carcinoma cells (Wang et al. 2010a; Yu et al. 2014). Cannabinoids (Δ9-THC) and its receptor agonist (JWH-015) inhibit autophagy induction by Akt–mTORC1 axis and AMPK stimulation, which suppresses the growth of subcutaneous xenografts. Studies have shown that Δ9-THC and agonist JWH-015 promote HCC liver cancer death through autophagy stimulation in human hepatoma cells and nude mice (Vara et al. 2011). As a novel antitumor agent, fangchinoline induces autophagic cell death in human hepatoma cells via the p53/sestrin2/AMPK signaling pathway (Wang et al. 2011). In addition, MLN4924 (a potent and selective small-molecule NEDD8 activating enzyme inhibitor) can inhibit the growth of hepatoma cells in vitro and in vivo by inducing autophagy. The autophagy is induced by binding Deptor (mTOR binding protein) to inhibit mTOR activity to induce autophagy in hepatoma cells (Luo et al. 2012). II.Autophagy inhibitors: autophagy inhibitors can enhance the cytotoxicity of therapeutic agents by removing the protective effect of autophagy and limiting the resistance of liver cancer treatment. (1) Chloroquine (CQ)/hydroxychloroquine (HCQ) and 3-MA: CQ and HCQ have been widely used as autophagy inhibitors. There have been many reports that CQ and HCQ sensitize various cancers to therapeutic drugs in vivo and in vitro. For example, inhibition of autophagy by CQ further increases oxaliplatin-induced apoptosis and increases chemosensitivity of hepatoma cells (Du et al. 2012). Ding et al. (2011) reported that the use of CQ to inhibit

Table 37.1 Autophagy inducers and inhibitors developed in in vitro and in vivo systems as well as in clinical trials

	Drugs	Test system
Autophagy inducer	Sirolimus (Rapamycin)	C57BL/6 mice, HepG2; mice
	Everolimus (RAD001)	Xenograft mice model, Hep3B, HepG2, and Huh7
	Everolimus + BEZ235	Hepatocyte cell line,mice, and Stage-I clinical trial
	Temsirolimus (CCI-779)	HepG2 and Huh7
	Bortezomib	Huh7 cells/FVB and transgenic mice
	SAHA/OSU-HDAC42	Hep3B, HepG2, and Huh7
	Panobinostat	Hep3B, HepG2, Huh7, and xenograft mice model
	SC-59	PLC5, Sk-Hep1, HepG2, and Hep3B
	MK-2206	SNU449, SNU378, and SNU475
	BEZ235	Hep3B and PLC/PRF/5
	Sorafenib	Hep3B, HepG2, and Huh7
	Regorafenib	HepG2 and Hep3B
	Nilotinib	Hepatocyte cell line and mice
	ABT-737	Huh7 and HepG2
	NPC-16	HepG2 and Bel-7402
	Berberine	MHCC97-L and HepG2
	MLN4924	Huh-7, Hep G2, and xenograftmicemodel
	OSU-03012	Huh7, Hep3B, and HepG2
	Δ9-THC/JWH-015	Hep G2, Huh-7, and xenograftmicemodel
	THC	Hepatocyte cell line, and mice
	Fangchinoline	HepG2 and PLC/PRF/5
	SB203580	HepG2, Hep3B, PLC/PRF/5, and Huh-7
	Melatonin	H22 and H22 mice models
	Cisplatin	Hepatocyte cell line, and mice
	Oxaliplatin	Hepatocyte cell line, and mice
	miR-100	Hepatocyte cell line, and mice
Autophagy inhibitor	3-MA	H22 and HepG2; xenograft mice model and PLC/PRF/5; SMMC7721
	Wortmannin	HCCC9810
	Bafilomycin A1	BEL7402, HepG2, Huh7, and SMMC-7721

(continued)

Table 37.1 (continued)

	Drugs	Test system
	Chloroquine(CQ)/HCQ	HepG2, Huh7, HA22T/VGH, and Mahlavu
	siRNA (siATG5,siBeclin1,siATG7,shBeclin1, and shATG5)	HepG2, H22; PLC/PRF/5; HA22T/VGH; Huh7,HCCLM3,MHCC97H, and SMMC7721
	MicroRNAs (mir-375, miR-101, and miR-199a-5p)	Huh7 and Hep3B, and xenograft mice

autophagy enhanced oxaliplatin-induced hepatoma cell death. Oxaliplatin combined with CQ significantly inhibited the growth of HLA tumors in vivo (Ding et al. 2011). Long-term sorafenib treatment has also been shown to trigger chemoresistance in HCC cells, which is associated with autophagy (Nishida et al. 2015). Sorafenib in combination with CQ has more pronounced tumor suppression in liver cancer cell lines and nude mouse models (Shi et al. 2011a, b). Similar results have also been found in other research groups (Shimizu et al. 2012). In liver cancer xenografts model, the combination of sorafenib and CQ significantly inhibited tumor growth compared with sorafenib alone. Currently, there are 25 ongoing clinical trials using CQ/HCQ alone or in combination with other drugs to treat tumors by targeted autophagy (http://www.clinicaltrials.gov/ct2/results?term=autophagy&Search=Search).

However, there have been no clinical trials of liver cancer cells. In xenograft liver tumor models, nude mice treated with a combination of CQ and sorafenib achieved higher levels than sorafenib alone. In addition, 3-methyladenine (3-MA) enhances anti-HCC therapy when used in combination with cisplatin, doxorubicin, and sorafenib (Sheng et al. 2018); (2) siRNA and shRNA: silencing of autophagy key gene via siRNA or shRNA has high specificity and selectivity, which can remarkably prevent the side effects of drugs. Chen et al. reported that autophagy can be inhibited by Beclin 1 knockdown and leading to increased cell death in liver cancer cell lines (Chen et al. 2011). Inhibition of autophagy by siRNA-silencing of ATG5 and Beclin 1 partially enhanced the response of HepG2 cells to MLN4924 treatment and increased cell death (Luo et al. 2012). Inhibition of autophagy via Beclin 1 silencing also enhances melatonin-induced cell death in H22 cells and increases the antitumor effect of melatonin (Liu et al. 2012). Collectively, silencing of specific ATG genes via siRNA inhibits autophagy to enhance chemotherapeutic drug-induced cell death, but it remains to be determined whether autophagy inhibition using siRNA actually affects the efficacy of liver cancer treatment. The use of viral vectors to carry shRNA or miRNA targeting key ATGs genes to in vivo inhibiting autophagy is a future development direction. Peng et al. designed a lentiviral vector-based hepatocarcinoma-specific autophagy shRNA targeting therapeutic vector. In vitro and in vivo (mouse liver cancer lung metastasis model) application showed that the system can effectively inhibit autophagy and suppress liver cancer lung metastasis (Peng et al. 2013a, b); (3) MicroRNAs (miRNAs) regulate cellular autophagy by

targeting multiple genes and pathways (Kim and Kim 2014). In liver cancer cells, microRNA acts on the ATG gene to regulate autophagy. Many miRNAs, including miR-101, miR-30a, miR-34a, miR-204, and miR-375, are autophagy inhibitors. For example, in liver cancer cells, mir-375 can effectively inhibit hypoxia-induced autophagy by directly acting on ATG7 (Chang et al. 2012). miR-375 mimic transfection can attenuate the protective effect of autophagy and impair the viability of liver cancer cells (Pastore et al. 2013). miR-101 can act on STMN, RAB5A and Atg4D to inhibit autophagy in hepatoma cells and synergize with doxorubicin and fluorouracil to induce tumor cell apoptosis, which can enhance the chemosensitivity of hepatoma cells (Xu et al. 2014; Li et al. 2014a). In cisplatin-treated liver cancer patients, miR-199a-5p was significantly decreased and miR-199a-5p down-regulated ATG7 to regulate autophagy-mediated resistance (Yamamura et al. 2014). Inhibition of autophagy with miRNAs is becoming an emerging focus. However, it should be noted that some microRNAs are responsible for inducing autophagy. miR-100 can inhibit the expression of mTOR and IGF-1R and promote HCC autophagy. Overexpression of miR-100 can lead to cell death, which can be inhibited by ATG7 silencing and CQ, providing a potential target for HCC treatment (Ge et al. 2014); and (4) Others: 20(S)-Ginsenoside Rg3 is a novel autophagy inhibitor that enhances doxorubicin treatment of liver cancer (Kim et al. 2014). Adriamycin-induced autophagy has a protective effect on liver cancer cells, and ginsenoside Rg3 can inhibit the autophagy. Rg3 can cooperate with doxorubicin to kill liver cancer cells. It is well tolerated and can be combined with doxorubicin to significantly suppress tumor in vivo.

It should be noted that the application of autophagy for treatment requires attention that the role of autophagy in tumors and therapeutic responses is complex. The double-edged sword function of autophagy may affect the efficacy of anticancer treatment in the opposite way. These considerations must be carefully considered when using autophagy to improve anticancer effect.

37.3 Autophagy and Alcoholic Liver Disease

It is reported that autophagy is important for protecting against ethanol-induced liver damage and hepatic steatosis. Autophagy plays a central role in removing damaged mitochondria and lipid droplet accumulation, eliminating the source of reactive oxygen species (ROS) (damaged mitochondria) and ROS amplifiers (lipids) required for ethanol-induced liver injury.

Early studies have suggested that autophagy may be inhibited in alcoholic liver disease. In the ethanol-infused rat liver, protein degradation was significantly inhibited. Electron microscopy of lysosomal components shows that the total volume of autophagosomes in hepatocytes was significantly smaller than that of normal cells (Poso et al. 1987). The mechanisms by which ethanol inhibits autophagy are that impaired hepatic lysosomal proteolytic activity, decreased lysosomal enzyme transport, damaged microtubule structure, and altered amino acids in the liver lead to

autophagy inhibition (Donohue 2009; Donohue et al. 1994). Alcohol-induced lysosomal damage is demonstrated by the increased lysosomal fragility regardless of changes in lipid metabolism and oxidative stress (Donohue et al. 1994). In ethanol-fed female rats, very low serum ethanol concentrations can cause functional damage to the lysosomal system. Another possible mechanism by which ethanol inhibits autophagy is the disruption of protein transport in the liver. Exogenous proteins are transported into hepatocytes by endocytosis and intracellular delivery of proteases to lysosomes can be inhibited by ethanol uptake. Acetaldehyde, a primary product of ethanol oxidation, inhibits the polymerization of tubulin to form microtubules, which are essential for autophagosome formation (Smith et al. 1989). Ethanol-induced autophagy inhibition may also be caused by changes in liver amino acid levels. In rats chronically ingested with ethanol, the level of leucine in the liver increased 1.4–1.8 times higher than that of the control animals (Bernal et al. 1993). Since leucine is the strongest autophagy-regulating amino acid, high levels of intrahepatic leucine may partially explain ethanol-induced autophagy inhibition.

But recent studies have shown that acute or chronic alcohol intake activates autophagy rather than inhibiting autophagy. Autophagy regulates intracellular lipid metabolism. Acute and chronic ethanol uptake can lead to activation of autophagy which activates clearance of damaged mitochondria and lipid droplets in the liver (Eid et al. 2013; Ding et al. 2010). Studies of chronic alcoholic fatty liver showed that chronic alcohol intake can lead to increased autophagy (Zeng et al. 2014). The histological feature of chronic alcoholic liver disease is the formation of Mallory-Denk bodies in hepatocytes. The Mallory-Denk body contains cytokeratin 8, cytokeratin 18, ubiquitin-positive protein aggregates and p62/SQSTM1. Autophagy is a key mechanism for cleaning up these protein aggregates. Acute ethanol intake, especially excessive intake, also significantly activates autophagy (Ding et al. 2010).

Some drugs can regulate autophagy in alcoholic liver disease. Autophagy induces can protect against liver damage caused by alcohol. For example, cannabis can inhibit alcohol-induced steatosis by increasing autophagy and inhibiting oxidative stress (Yang et al. 2014). Globular adiponectin (gAcrp) regulates ATG5 expression, promotes autophagy, reduces apoptosis, and inhibits ethanol-induced hepatocyte death. Alcohol inhibited ATG5 expression in primary rat hepatocytes and HepG2 cells, which can be reversed by pretreatment with gAcrp (Nepal et al. 2014). Autophagy inhibitors can aggravate alcoholic liver disease. In mouse model of alcoholic liver disease, autophagy inhibitor wortmannin can aggravate ethanol-induced fatty liver at higher doses (Zeng et al. 2012).

37.4 Autophagy and Nonalcoholic Fatty Liver Disease (NAFLD)

Nonalcoholic fatty liver disease (NAFLD) is a common liver metabolic disease, including simple fatty liver, nonalcoholic steatohepatitis (NASH) and related liver fibrosis, cirrhosis and liver cancer. The treatment of NAFLD is currently attracting

more and more attention. The liver is the second largest lipid storage warehouse in addition to fat. As an important regulation mechanism of endogenous fat metabolism other than lipase, autophagy is closely related to the pathogenesis of NAFLD. Previous studies have shown that autophagy can reduce lipid accumulation and alleviate disease progression in the early stages of NALFD while it can assist the activation of hepatic stellate cells (HSCs) to promote liver fibrosis development in the later stages.

Lipid deposition is the key of NAFLD. Autophagy can degrade subcellular organelles including lipid droplets. Autophagy dysfunction is one of the important causes of NAFLD (Lin et al. 2013; Li et al. 2017; Valenti et al. 2009; Zhang et al. 2016). Studies have shown that autophagy is impaired in NAFLD patients, NAFLD mouse models, and hepatocytes with lipid overloaders. Autophagy can reduce or prevent progression of NAFLD (Gonzalez-Rodriguez et al. 2014). In normal human liver, the expression of autophagy-related protein LC3 is strong and the ubiquitin-binding protein P62 is weak. In the liver of patients with steatosis, the expression of LC3 gradually decreases with the severity of the disease and P62 is enhanced (Kwanten et al. 2014). In the early stages of NAFLD, autophagy can reduce hepatic lipid droplets and reduce steatosis by degrading intracellular lipid droplets. After autophagy is inhibited, the action of lipolysis is attenuated and the process from accumulation of lipid droplets to steatosis cannot be prevented, which in turn aggravates the disease and forms liver fibrosis. Lipid accumulation and increased fat content in the liver is observed after 18 weeks of high-fat diet in ATG knockout mice. Rapamycin induced autophagy significantly reduced the intrahepatic fat content and improved insulin sensitivity (Lin et al. 2013). Branched-chain amino acids (BCAA) inhibit liver autophagy by activating mTOR. BCAA and high-fat feeding (HFD) mouse models have shown that BCAA feeding can significantly reduce the body weight of HFD-fed mice and reduce intrahepatic triglyceride levels, liver damage, and development of NAFLD (Zhang et al. 2016). These studies indicate that a decrease in the ability of degrading intrahepatic lipid droplets due to attenuated autophagy causes a sustained increase in lipid droplets and progression to steatosis. Li et al. (2017) found that 1,25 bishydroxylated vitamin D3 can prevent hepatic steatosis caused by HFD or free fatty acids. The mechanism is that 1,25(OH)2D3 upregulates ATG16L1 expression which mediates autophagy activation and reduce intrahepatic lipid accumulation, thereby reduce fatty degeneration and liver inflammation (Saitoh et al. 2008). The cause of impaired autophagy in hepatocytes during the development of NAFLD is associated with increased ER stress (Gonzalez-Rodriguez et al. 2014). However, the specific mechanism is still unclear. In the late stage of NAFLD, autophagy plays an opposite role. Autophagy activates hepatic stellate cells (HSCs) to provides energy and causes liver fibrosis. Hepatic stellate cells (HSCs) are rich in lipid droplets, which generate a large number of extracellular matrices, synthesize collagen and produce actomyosin, which induce liver fibrosis, and the activation of HSCs requires a large amount of ATP (Blaner et al. 2009). A mouse model of liver fibrosis induced by carbon tetrachloride (CCl4) showed that the expression of LC3 was significantly increased and the level of autophagy was increased. The autophagy inhibitor bafilomycin A1 (bafilomycin A1) increase the intracellular lipid droplets, this corresponds to the lipotoxic effect of autophagy in the early stages of NALFD

(Thoen et al. 2011; Hernandez-Gea et al. 2012). Models of liver injury induced by CCl4 and thioacetamide (TAA) in ATG7 knockout mice showed that HSCs activation was suppressed after autophagy was inhibited, and fibrosis was alleviated (Thoen et al. 2011; Hernandez-Gea et al. 2012). These indicate that the energy required for HSCs activation is derived from autophagic lipid droplets in hepatocytes. Inhibition of autophagy can effectively reduce the activation of HSCs and thereby reduce liver fibrosis. A feature of nonalcoholic steatohepatitis is elevated plasma free fatty acid (FFA) levels. Free fatty acid-induced lipotoxicity plays a crucial role in the progression of NAFLD. Free fatty acids induce autophagy and autophagy plays a protective role in this process. Studies have shown that autophagy in human NASH and HFD-fed mice significantly increased. The palmitic acid treatment can significantly increase autophagy in SMMC-7721 cells or HepG2 cells. Palmitic acid may induce hepatocyte autophagy through activation of JNK2 and PKCα. 3MA, chloroquine or ATG5 silencing increase apoptosis after PA treatment, while rapamycin pretreatment induces significantly decreased PA-induced apoptosis, indicating the protective effect of autophagy in PA-induced hepatocyte apoptosis (Cai et al. 2014; Tu et al. 2014).

Hepatic lipid metabolism disorders are also associated with another form of autophagy, chaperone-mediated autophagy (CMA) (Schneider et al. 2014). CMA autophagy defects can lead to fatty liver. Key enzymes in carbohydrate and lipid metabolism are usually degraded by CMA. CMA deficiency impaired the degradation and forms of metabolic disorders. Liver CMA-deficient mice showed that obstruction of CMA results in fatty liver. NAFLD-related states such as hyperinsulinemia and high energy supply inhibit autophagy itself, which in turn increases hepatic fat, reduces insulin sensitivity, and makes cells prone to death. Upregulation of autophagy may disrupt the negative feedback vicious circle by reducing insulin resistance and lowering triglyceride levels to protect cells from oxidative damage and cell death. Recent studies have shown that mice fed a high-fat diet with autophagy enhancer rapamycin or carbamazepine can reduce liver and blood triglyceride levels and liver damage (Lin et al. 2013). Caffeine is a strong stimulant for autophagy. It can burn liver fat by inducing lipid autophagy and mitochondrial β oxidation. In HFD-fedNAFLD mouse model, caffeine significantly reduced fatty liver (Sinha et al. 2014; Ding 2014a). Caffeine may inhibit PI3K-AKT by activating the Ulk1 complex and then inhibit mTOR to activate autophagy. Autophagy selectively removes excess LDs to produce free fatty acids. The decrease if mTOR induces the nuclear translocation of TFEB. TFEB upregulates autophagy and lysosomal-associated genes as well as PGC-1α and PPARα expression, increasing mitochondrial β-oxidative combustion of free fatty acids. Therefore, caffeine prevents fatty liver by synergistically inducing lipophagy and mitochondrial β oxidation (Ding 2014a). Autophagy inducer carbamazepine reduces fatty liver and improves insulin sensitivity in hepatocytes and mice (Lin et al. 2013). Rapamycin induces autophagy, reduces hepatotoxicity and fatty liver (triglyceride levels), and improves insulin sensitivity (Lin et al. 2013; Sinha et al. 2014). However, it should be noted that excessive autophagy induction induces autophagic cell death (Yamaguchi et al. 2007; Amir and Czaja 2011). Resveratrol also regulate autophagy and improves liver damage caused by NAFLD.

Wild-type and heterozygous knockout of autophagy media ULK1 mice was fed with a high-fat diet to establish nonalcoholic fatty liver disease (NAFLD) mouse model. Resveratrol regulates autophagy and NF-κB pathway to improve liver damage caused by NAFLD (Li et al. 2014b). tBHQ can induce autophagy to prevent fatty toxicity caused by saturated fatty acids (Li et al. 2014c). Saturated fatty acids induce hepatocyte death in rats, which is associated with oxidative stress mechanisms. Nrf2 is a major transcriptional regulator of cellular antioxidant defense enzymes. Activation of Nrf2 is considered as an effective strategy to trigger oxidative stress and cell damage. Epigallocatechin gallate (EGCG) is a major polyphenol in green tea and has an anti-fatty liver effect. Recent studies have shown that EGCG induces autophagy through the AMPK pathway, increases lipid droplets and lipid clearance, and promotes lipid metabolism. EGCG treatment reduced fatty liver in mice fed a high-fat (Zhou et al. 2014). In obese diabetic rats, Roux-en-Y gastric bypass reduces excessive lipid accumulation in the liver by upregulating hepatocyte autophagy (Ejaz et al. 2014). Appropriate amount of rapamycin can increase the autophagy activity of liver tissue and reduce the steatosis. The steatosis is reduced from 0.16 (7/45) to 0.12 (5/43) after Rapamycin application, and the lymphocytic infiltration of the liver reduced from 0.24 (11/45) to 0.07 (3/43) after Rapamycin application (Zhang et al. 2014b). The use of the acid sphingomyelinase (ASMase) inhibitor amitriptyline can protect hepatic steatosis, fibrosis, and liver damage induced by a high-fat diet (Fucho et al. 2014).

The molecular mechanism of autophagy in NAFLD is unknown. Inokuchi-Shimizu et al. reported that TAK1 regulates hepatic lipid metabolism through the AMPK/mTORC1 axis and PPARα activity. TAK1 protein mediates autophagy and fatty acid oxidation to prevent fatty liver development (Inokuchi-Shimizu et al. 2014). Fasting mouse with hepatocyte-specific TAK1 knockout showed severe fatty liver. mTORC1 inhibition suppressed the spontaneous liver fibrosis and liver cancer in the TAK1 animals. Acid sphingomyelinase (ASMase) regulates autophagy and lysosomal membrane permeability. The ASMase knockout mice confirmed that the primary mouse hepatocytes of ASMase-/- mice had low autophagy activity and defects in autophagy. Diet-induced NASH model study showed that the use of the ASMase inhibitor amitriptyline protects hepatic steatosis, fibrosis, and liver damage (Fucho et al. 2014).

Autophagy is gaining attention as a new therapeutic target due to its important role in the development of NALFD. In the early stage of NAFLD, induction of enhanced autophagy contributes to lipid degradation in the liver and alleviates disease progression. In the late stage of NAFLD, inhibition of autophagy can inhibit the progression of the disease. However, the research on autophagy in the field of NAFLD is still in its infancy, and requires further research.

37.5 Autophagy and Liver Fibrosis, Cirrhosis

Hepatic fibrosis is defined as the excessive proliferation and abnormal deposition of extracellular matrix (ECM) components in liver tissue, leading to pathological changes in liver structural and functional abnormalities. Liver fibrosis can be caused by a variety of reasons, including hepatitis B/C, alcoholic hepatitis, NAFLD, etc. Liver fibrosis can develop into cirrhosis when effective treatments is not administrated.

Autophagy is involved in the development of liver fibrosis and cirrhosis (Song et al. 2014; Su et al. 2014). A direct evidence is that mouse hepatocyte-specific ATG5 knockout autophagy defects can lead to increased hepatic apoptosis, inflammation, and fibrosis (Ni et al. 2014). The mechanism of autophagy's involvement in liver fibrosis and cirrhosis is not fully understood. Its role in liver fibrosis is complicated. It can promote the formation of liver fibrosis (for example, activation and induction of hepatic stellate cells, macrophages, hepatic sinusoidal endothelial cells, etc.), while it can also inhibit the progression of liver fibrosis (for instance, degrade abnormal proteins such as ATZ by autophagy). Hepatic stellate cells (HSC) are the main cells for the synthesis of ECM in the liver. Its activation is the core of the occurrence and development of liver fibrosis. HSC is an interstitial cell that synthesizes and secretes extracellular matrix (ECM) and produces collagenase. The activation of HSC requires energy consumption. Autophagy provides energy for the activation of HSC by degrading lipids, which in turn mediates the activation of HSC and plays an important role in the development and progression of liver fibrosis (Thoen et al. 2011; Lee and Friedman 2011; Song et al. 2014). Studies have shown that autophagy activity of liver fibrosis is significantly enhanced. In the model of liver fibrosis induced by carbon tetrachloride, the expression of autophagy-related gene LC3B was significantly increased, indicating that autophagy activity was significantly enhanced in liver fibrosis. The autophagy activity of HSC isolated from fibrotic liver tissue of patients with hepatitis B also significantly enhanced (Chen et al. 2010; Hernandez-Gea et al. 2012). In chronic liver injury model, mouse HSC-specific ATG7 knockdown can reduce liver fibrosis, and inhibition of autophagy can reduce proliferation and activation of primary HSC in mice (Krenkel and Tacke 2017). The cytoplasm of HSC at rest is filled with lipid droplets, and the lipid droplet volume is reduced after HSC activation (Testerink et al. 2012). When the lipid droplet-rich HSC activation is converted to myofibroblasts, the autophagic flux increases. Treatment with autophagy inhibitor 3MA or knockout of autophagy-related gene Atg5 inhibited autophagy and resulted in increased triglyceride content in lipid droplets, inhibition of hepatic stellate cell activation, decreased fibrogenesis, reduced fatty acid β oxidation levels, and diminished fatty acid synthesis (Thoen et al. 2011; Blommaart et al. 1997; Heaton et al. 2010; Hernandez-Gea et al. 2012). In animal model of liver fibrosis established by carbon tetrachloride (CCl4) or thioacetamide (TAA), it was found that the autophagy activity of HSC in liver fibrosis mice was significantly increased, and inhibition of autophagy by 3-MA or CQ reduce fibrosis. Furthermore, ATG7 knockout autophagy-deficient mice showed a significant reduction in liver fibrosis when

liver fibrosis was induced by CCl4 or TAA. Another study showed that autophagy-mediated lipid degradation during HSC activation is a key source of energy (Gracia-Sancho et al. 2014). These all suggest that autophagy is a source of HSC activation. The specific mechanism by which autophagy activates HSC is not fully understood. Phospholipase D1 (Phospholipase D1) is known to reduce hepatic stellate cell type I collagen levels by inducing autophagy (Seo et al. 2014). Phospholipase D1 (PLD1) catalyzes the hydrolysis of phosphatidylcholine to produce phosphatidic acid (PA) and choline. Adenovirus-mediated overexpression of PLD1 activates autophagy in hepatic stellate cells (HSC) cells and reduces type I collagen levels. Bafilomycin or ATG7 silencing can inhibit type I collagen accumulation in hepatic stellate cells induced by adenovirus-mediated PLD1 overexpression. Nrf2 was also found to be involved (Ni et al. 2014). Mouse hepatocyte-specific ATG5 gene knockdown results in increased hepatocyte apoptosis, inflammation, and fibrosis. Nrf2 knockout significantly eliminated these pathological changes, indicating that Nrf2 plays an important role. Transforming growth factor β1 (TGFβ1) reduces hepatic stellate cell apoptosis by activating autophagy. TGFβ1 treatment induced autophagy in rat HSC cells. Bafilomycin A1 and LC3 knockdown inhibited apoptosis and proliferation (Fu et al. 2014). Shen et al. reported that astaxanthin protects liver fibrosis by down-regulating TGF-β1 expression and down-regulating autophagy (Shen et al. 2014). Thomes et al. (2012) found that the autophagy behavior of HSC can promote the proliferation of HSC cells through hypoxia-inducible factors and promote the collagen production by TSAβ1/Smad signaling. Theoretically, the autophagy inhibitors may be used to treat liver fibrosis by inhibiting the activation of HSC. The development of drugs that specifically inhibit autophagy of HSC can delay or reverse liver fibrosis. In addition to the involvement of hepatic stellate cells in the process of liver fibrosis, other cells are involved in the process of liver fibrosis. Increased autophagy activity of sinusoidal endothelial cells and macrophages is beneficial in reducing liver fibrosis, especially in the early stages of the disease. Liver macrophages lacking autophagy promote liver inflammation and liver fibrosis by enhancing the mitochondrial ROS-NF-κB-IL-1α/β pathway (Sun et al. 2017). Hepatic sinusoidal endothelial cells also play an important role in the development of liver fibrosis. Endothelial cells maintain vascular tone and keep HSC at rest. It has been demonstrated that autophagy is extremely important to maintain the homeostasis of endothelial cells. In animal models of liver fibrosis, loss of autophagy in endothelial cells can aggravate fibrosis (Ruart et al. 2019). Autophagy can also affect liver fibrosis through hepatocytes in addition to hepatic stellate cells, macrophages, and endothelial cells, which partly explains the role of autophagy in different types of liver disease. Hepatitis B and hepatitis C are important causes of liver fibrosis. HBV and HCV activate autophagy in hepatocytes, and autophagy is involved in promoting the replication of HBV and HCV. Hepatocytes containing HCV or replicating HCV produce fibrogenic stimulating factors that stimulate HSCs to form liver fibrosis (Song et al. 2014). Autophagy plays a role in liver fibrosis caused by abnormal protein aggregation such as α-1-antitrypsin deficiency or fibrinogen storage disease. Autophagy can degrade the aggregation of AT in the endoplasmic reticulum and reduce hepatocyte apoptosis and liver damage and subsequently plays a protective role in reducing liver fibrosis and cirrhosis.

Using autophagy inducer carbamazepine to induce autophagy may reduce protein accumulation and liver fibrosis (Pastore et al. 2013; Puls et al. 2013; Hidvegi et al. 2010).

The dual role of autophagy in liver fibrosis makes autophagy a challenging for treatment of liver fibrosis. Individual treatments need to be applied in combination with different types of liver disease and different degrees of liver fibrosis. Some autophagy-regulating drugs can usually be used to reduce liver fibrosis and cirrhosis, such as the use of astaxanthin to down-regulate autophagy to protect liver fibrosis (Shen et al. 2014). In vivo study showed that dimethyl α ketoglutaric acid can reduce Carbon tetrachloride-induced liver fibrosis by inhibiting the autophagy activity of HSC (Zhao et al. 2016a). The autophagy inhibitor bapromycin or ATG7 over-expression also inhibits the accumulation of type I collagen in HSC (Seo et al. 2014). ATG5 knockdown or 3-methyladenine can significantly promote HSC apoptosis (Hao et al. 2016). So far, there are many potential autophagy-related drugs for the treatment of liver fibrosis, but there is no one drug available for clinical application due to the complexity and cell specificity of autophagy during liver fibrosis (Tacke and Weiskirchen 2018).

37.6 Autophagy and Liver Damage

Hepatic ischemia-reperfusion injury (IRI) is an important cause of liver damage during surgical procedures such as liver transplantation and general liver resection. It is also a major cause of liver failure after liver transplantation. Recent studies have shown that autophagy plays a role in liver damage caused by ischemia-reperfusion injury. In the liver-specific ATG5 knockout autophagy-deficient mice, liver regeneration after partial hepatectomy was severely impaired with reduced postoperative mitosis. Analysis showed that hepatectomy caused a decrease in intracellular ATP and β oxidation, and cell mitochondrial damage. Liver resection enhances accumulation of p62 and ubiquitin in the liver. The intracellular protein and organelle reorganization are impaired during liver regeneration (Toshima et al. 2014).

The mechanism of hepatic IRI is complex. Although it has not yet been fully elucidated, it is generally considered to be excessive production of reactive oxygen species (ROS), calcium overload, endoplasmic reticulum stress, and mitochondrial damage. Mitochondrial damage and lipid peroxidation are the central events in the development of IRI. Theoretically, autophagy selectively removes damaged mitochondria and lipids, reduces ROS production and protects the liver (Go et al. 2015). It has been demonstrated that autophagy can counteract ischemia-reperfusion injury. Calcium overload and calpain-activated injury can cause liver damage after hepatic ischemia-reperfusion. Autophagy inhibition by chloroquine and wortmannin can aggravate liver damage, while heme oxygenase-1 (HO-1) protects ischemia-reperfusion injury by enhancing autophagy (Yun et al. 2014). There are a large number of autophagosomes in the liver cells after hepatic ischemia-reperfusion. Inhibition of autophagy by inactivation of HO-1 results in hepatic injury caused by liver

ischemia-reperfusion in vivo. Hemin-induced autophagy also protects rat hepatocytes from ischemia-reperfusion injury in vitro. The siRNA silencing of HO-1 inhibits autophagy to eliminate this effect (Wang et al. 2014). Melatonin (MLT) inhibits mTOR-dependent autophagy during hepatic ischemia/reperfusion (Kang et al. 2014). Melatonin is a potent endogenous antioxidant that has a beneficial effect on hepatic ischemia-reperfusion injury. Murine liver ischemia-reperfusion increases autophagy and melatonin can reduce it. Ischemia-reperfusion reduces the phosphorylation of 4E-BP1 and 70s6k downstream of mammalian mTOR and mTOR signaling pathways, while MLT can increase mTOR, 4E-BP1,, and 70s6k phosphorylation. Pretreatment with rapamycin reverses the effects of MLT on autophagy flux and mTOR pathway. Melatonin down-regulates autophagy via the mTOR signaling pathway, which may contribute to the protective effect of melatonin on hepatic ischemia-reperfusion injury. Another more common research model is sepsis liver injury. C57BL/6 mice were induced by cecal ligation and perforation. The mice were given autophagy enhancer carbamazepine (CBZ) and autophagy inhibitors (3-MA and CQ). Carbamazepine reduces cell death, inflammation and liver damage, and improves survival in mice, where as autophagy inhibitors exacerbated these effects (Lin et al. 2014). Carbon monoxide (CO) also protects against liver sepsis by autophagy (Lee et al. 2014). C57BL/6 mice were induced by cecal ligation and perforation to induce sepsis. CO enhanced autophagy and phagocytosis and increased survival in mice. Hepatic injury model was induced by lipopolysaccharide (LPS) stimulation. LPS induced p62-mediated (not Beclin1-mediated) autophagy clearance in hepatocytes, clearing aggregated or misfolded proteins, and protecting energy balance under stress (Chen et al. 2014a). Autophagy protects liver cells against TNF-induced liver injury. In hepatocyte-specific ATG7 knockout mice, the levels of serum alanine aminotransferase in mice treated with D-galactosamine (GalN) and lipopolysaccharide (LPS) increased. Hepatocyte autophagy defects make hepatocytes more sensitive to GalN/TNF injury with increased tissue damage, cell apoptosis and cell death. Overexpression of Beclin 1 to increases autophagy can prevent GalN/LPS damage. Autophagy mediates TNF toxicity by blocking the activation of Caspase8 and the mitochondrial death pathway in vivo, suggesting that autophagy is a therapeutic target for TNF-dependent liver injury (Ding 2014b). Schisandrin A protects hepatocyte against acute liver injury induced by galactosamine through activating autophagy (Lu et al. 2014b). Usnic acid is associated with acute hepatic failure. Autophagy protects against hepatic cytotoxic damage induced by usnic acid. After inhibition of autophagy by 3-MA or CQ or ATG7 silencing, usnic acid results in aggravated hepatocyte apoptosis and decreased cell survival (Chen et al. 2014b). Acetaminophen (APAP) induces liver injury in mice, and total saponins from Rosa laevigata Michx fruit can inhibit inflammatory and apoptosis by inducing autophagy against acetaminophen-induced liver injury (Dong et al. 2014).

Due to the prosurvival role of autophagy in liver injury, autophagy induction is used to alleviate liver damage. Autophagy inducers, including cisplatin, lithium, melatonin + TMZ, and rapamycin, can induce autophagy and reduce liver damage (Gracia-Sancho et al. 2014). But the application needs to be cautious. The degree

of activity of autophagy in IRI is often puzzling. In the model of hepatic ischemia-reperfusion injury, autophagy changes were reported differently. Some studies have shown that intracellular autophagy levels are up-regulated (Przyklenk et al. 2012; Zhou et al. 2016; Hong et al. 2016; Li et al. 2016b), while other studies have shown that autophagy levels are down-regulated (Kim et al. 2008; Zhou et al. 2016; Zhao et al. 2016b).The changes in autophagy levels have also leaded to different results. Some studies have shown that autophagy induction result in alleviation of ischemia-reperfusion injury, increased metabolism capacity and cell viability, and organs under ischemia-reperfusion conditions were protected (Kim et al. 2008; Zhou et al. 2016; Zhao et al. 2016b). In contrast, ischemia-reperfusion injury was aggravated after induction of autophagy (Li et al. 2016b; Hong et al. 2016; Ma et al. 2011). These results are puzzling and some scholars have suggested that the mechanism may be associated with the difference of injury at different stages of IRI. Short time of ischemia (30 min) generally up-regulated the level of autophagy, while long-term ischemia (more than 90 min) leads to a down-regulation of autophagy. In the case of hepatic IRI, moderate upregulation of autophagy can clear abnormal intracellular structural lipids and damage mitochondria, reduce the release of mitochondrial pro-apoptotic factors and ROS production, and maintain intracellular lipid homeostasis, energy metabolism, and cell membrane stability to promote cell survival. However, excessive stimulation beyond the range of cell tolerance can lead to autophagic damage and even autophagic cell death. At this circumstance, the use of autophagy inhibitors can reduce the level of autophagy in hepatocytes to promote cell survival. The molecular mechanism of autophagy to regulate liver injury is unclear. Recent studies suggest that it is associated with NRBF2-mediated PI3K-III regulation of autophagy (Lu et al. 2014a).

37.7 Autophagy and Viral Hepatitis

Autophagy is involved in the pathogenesis of viral hepatitis. They often interact with each other. The hepatitis virus can induce autophagy. For example, both hepatitis B and hepatitis C virus can up-regulate Beclin-1 expression and induce autophagy (Abdoli et al. 2018). Autophagy can in turn promote the replication of hepatitis B and hepatitis C (Song et al. 2014).

HBV infection is the most important cause of liver cancer. There are two main mechanisms for HBV-related liver cancer. First, the immune response of enhanced T cells leads to necrosis, inflammation, and regeneration of liver cells, which ultimately leads to liver cancer. Second, in the case of HBV infection, enhanced endoplasmic reticulum stress, and oxidative stress can stimulate growth and survival signal transduction pathways and further induce multiple mutations through the generation of free radicals and activation of stellate cells. As a double-stranded DNA virus, hepatitis B virus can regulate autophagy and enhance self-replication, suggesting that targeting autophagy pathway may treat hepatitis B (Sir et al. 2010). HBV can induce autophagy in hepatocytes, and autophagy can in turn enhance HBV replication. This

phenomenon widely present in human HBV-infected individuals (Pant et al. 2016). The specific mechanisms for this phenomenon are currently controversial (Pant et al. 2016). Recent evidence show that autophagy is activated during HBV infection and enhanced autophagy promotes HBV replication in liver cancer cells and mice (Li et al. 2011; Tian et al. 2011). In Atg5-deficient transgenic mice, autophagy is restricted and the HBV DNA load and serum HBeAg/HBsAg concentrations are significantly decreased (Li et al. 2011; Tian et al. 2011). In addition, HBV transfected liver cancer cells can induce autophagy, which further enhances viral replication by binding of HBx protein to pi3kc3 (Sir et al. 2010). Tang et al. report that HBV transfection can activate the expression of the autophagy gene Beclin 1 protein and thereby enhance autophagy (Tang et al. 2009). In addition, autophagy can also be induced by small coat proteins (SHBs) through UPR (Eisenberg-Lerner et al. 2009). These findings indicate that the HBV virus utilizes the autophagy pathway for viral replication and autophagy pathway can serve as a new target for the treatment of HBV infection.

The interaction between hepatitis C virus and autophagy has also been reported. Hepatitis C virus RNA replication can induce UPR and C/EBP homologous protein (CHOP) expression and thereby activate autophagy by promoting autophagosome formation (Sir et al. 2008; Ait-Goughoulte et al. 2008). Some ATGs, including Beclin 1, ATG4b, ATG5, and ATG12, are involved in the translation of viral mRNA and replication initiation (Dreux et al. 2009). On the other hand, RNA replication can block the maturation of autophagosomes and autophagosomes by promoting their replication through utilizing autophagosome content (Sir et al. 2008). In addition, HCV can prevent itself from being identified by the autophagy mechanism (Alavian et al. 2011). Rautou et al. report that HCV proteins are not co-localized with autophagic vacuoles, suggesting that HCV can escape the host cell defense system (Rautou et al. 2010). The autophagy inhibitor chloroquine (CQ) can inhibit hepatitis C virus replication in hepatocytes (Mizushima et al. 2011). Inhibition of autophagic proteolysis is shown to be a novel therapeutic strategy for the treatment of this type of viral hepatitis (Mizui et al. 2010). The differential effects of autophagy on a variety of Hepatitis C virus-infected cell signaling pathways suggest that autophagy is required for the balance of HCV and host cell interactions and is involved in HCV-associated liver disease. However, how the HCV virus activates autophagy to act on viral growth and proliferation is still unclear (Ke and Chen 2014).

Autophagy-regulating drugs may play a role in the treatment of viral hepatitis. For example, 3-MA can inhibits HBV replication and reduces nuclear transcription of HCV RNA (Shinohara et al. 2013; Mizui et al. 2010; Su et al. 2011; Tang et al. 2009; Li et al. 2011). CQ and HCQ can inhibit HBV replication and enhance the antiviral effect of IFN-a (Mizui et al. 2010; Chandra et al. 2014). Ferrocquine can inhibit HBV replication and reduces entry of HBV into cells (Vausselin et al. 2013). Bafilomycin A1 can reduce HCV RNA nuclear transcription (Sir et al. 2010).

37.8 Autophagy and Hypofibrinogenemia

The quality control of protein by endoplasmic reticulum (ER) functions through the secretory pathway to eliminate abnormal proteins. The R375W mutation in the fibrinogen g-chain causes molecular misfolding that is abnormally recognized by ER and degraded by ER-related protein degradation (ERAD), which results in fibrinogenemia. Accumulation of fibrinogen variants in the liver ER leads to cirrhosis. Although the soluble form of this mutant can be degraded by the proteasome through ERAD, it is still necessary for autophagy to degrade the too much soluble abnormal protein and those most important insoluble aggregates in the ER (Kruse et al. 2006b). Regarding this disease, autophagy is thought to be associated with ER protein quantity control. In circumstance that the proteasome and ERAD are inhibited, autophagy is significantly activated and the endoplasmic reticulum stress is addressed by the unfolded protein response (UPR) pathway (Ding et al. 2007; Ding and Yin 2008). Under these circumstances, autophagy has been found to play an important role in alleviating ER stress by eliminating misfolded protein accumulation due to ERAD blockade. In this way, autophagy protects cells from cell death caused by endoplasmic reticulum stress.

37.9 Conclusion

Collectively, autophagy participates in the physiological metabolism of hepatocytes and plays an important role in the development of many liver diseases. Exploring the potential value of autophagy in the treatment of liver disease is undoubtedly a future direction. With the clarification of the exact role and mechanism of autophagy in liver pathophysiology, autophagy is hoped to improve the prevention and treatment of liver diseases and benefit more liver disease patients.

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Chapter 38
Autophagy and Gastrointestinal Diseases

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Abstract Normal gastrointestinal physiology is fundamental for all the living beings. Gastrointestinal diseases mainly include gastrointestinal motility disorders, infectious inflammation (such as *Helicobacter pylori* infection, cholera, and intestinal parasites), non-infectious inflammation (such as chronic gastritis and Crohn's disease), and gastrointestinal cancers. In addition, intestinal microbial disorder is also an important cause of intestinal diseases, so intestinal microecological treatment (fecal microbiota transplantation) is an important mean of treating gastrointestinal diseases. In recent years, the role of autophagy in gastrointestinal diseases has been studied extensively. Autophagy is observed under various pathological processes of the gastrointestinal tract. For example, it has been demonstrated that autophagy plays an important role in maintaining the homeostasis and integrity of intestinal epithelium. Additionally, autophagy regulates host response to *H. pylori* infection and development of gastrointestinal cancers. Therefore, we will discuss pivotal roles of autophagy in various gastrointestinal diseases and analyze the underlying molecular mechanisms, which may provide new therapeutic targets applicable for the treatment of gastrointestinal diseases.

Keywords Autophagy · Chronic atrophic gastritis · Helicobacter pylori · Chronic non-atrophic gastritis · Inflammatory bowel disease · Gastrointestinal infections · Intestinal microecology · Gastrointestinal motility disorders

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38.1 Overview

The gastrointestinal system consists of stomach, small intestine, and large intestine. It is an important place for digesting nutrients and excreting metabolic products. The stomach is an important organ for storing and digesting food. Pepsin in the gastric juice digests a portion of the protein in the food, and the stomach acid in the stomach provides the acidic environment necessary for the function of the pepsin. The food that enters the small intestine from the stomach is further decomposed with the aid of pancreatic-derived pancreatic enzymes and liver-derived bile, and a large amount of fluff in the small intestine increases the absorption area of nutrients. The main function of the large intestine is to further absorb water and dielectrics, forming and storing feces. Since the gastrointestinal tract is directly connected to the outside world, intestinal microbes, which are called the ninth largest organ of the human body, are anchored in the gastrointestinal tract.

The normal function of digestive system is crucial for human beings. Gastrointestinal diseases mainly include infectious inflammation (such as intestinal parasites, *H. pylori* infection, and cholera), non-infectious inflammation (such as Crohn's disease and chronic gastritis), gastrointestinal motility disorders, and gastrointestinal cancers. In addition, turbulence of intestinal microbial composition also plays an important role in intestinal diseases. Importantly, autophagy is observed under various pathological processes of the gastrointestinal tract. For example, it has been demonstrated that autophagy is crucial for maintaining the homeostasis of intestinal epithelium. Meanwhile, autophagy regulates host response to *H. pylori* infection and development of gastrointestinal cancers.

38.2 Autophagy and Non-infectious Inflammation of Gastrointestinal Tract

38.2.1 Autophagy and Chronic Atrophic Gastritis

38.2.1.1 Changes in Autophagy During the Development of Chronic Atrophic Gastritis

Chronic atrophic gastritis (CAG) is a type of chronic gastritis characterized by a decrease in the intrinsic glandular mucosa. The main clinical manifestations are upper abdominal pain, abdominal distension, abdominal discomfort, loss of appetite, and occasionally anemia, weight loss or diarrhea. Chronic atrophic gastritis belongs to a pathological concept, including metaplastic atrophy and non-metaplastic atrophy. The former refers to the glandular glands that are replaced by intestinal metaplasia or pseudo-pyloric gland metaplasia. Replacement with fibrous or fibromuscular tissue, or infiltration of inflammatory cells causes a decrease in the number of intrinsic glands. CAG is an intermediate stage in the conversion of normal gastric mucosa

into cancer. It is a benign lesion. If it is not controlled, it may increase the chance of transformation into gastric cancer. In 1978, the World Health Organization listed CAG as a precancerous lesion of gastric cancer, and the cancer rate is 1–3%.

The cause of chronic atrophic gastritis mainly includes all pathological processes that can cause gastric mucosal damage and lead to a decrease in the number of glands and intestinal metaplasia. *H. pylori* (HP) infection and autoimmune injury are the main causes of chronic atrophic gastritis. Autophagy plays an important role in the process of chronic atrophic gastritis.

HP is one of the most common human gastrointestinal pathogens, mainly colonized in gastric mucosal epithelial cells. 50–60% of the world's population is infected with this disease. HP infection has been considered as an infectious disease. It is recognized as a class I carcinogen, and HP infection is a major factor in the development of chronic atrophic gastritis and the progression to intestinal and cancerous development. Studies have shown that autophagy changes throughout the process of precancerous lesions such as chronic atrophic gastritis, intestinal metaplasia, and dysplasia caused by HP. HP has a two-way regulation of autophagy. After HP infection, vacuolar toxin (vacuolating cytotoxin A, VacA), HP secretion-associated antigen (HP0175), and HP flagella can activate autophagy in gastric epithelial cells, and autophagy can degrade VacA and reduce HP toxicity to cells, which suggests that autophagy is a protective mechanism after HP infection. Some studies have also pointed out that the expression of autologous activating gene ATG16 is significantly downregulated after HP infection, and a variety of miRNAs are also involved in down-regulation of autophagy, which creates a suitable environment for HP infection, which leads to inflammation and precancerous lesions.

Autoimmune gastritis refers to diffuse atrophy of the stomach in the context of CD4+ T cell-mediated autoimmune diseases, specific autoantibodies (e.g., anti-internal factors antibody, anti-parietal cell antibody, anti-gastrin-secreting cells antibody) are the main cause of gastric gland atrophy; and the incidence rate in China is low. Recent studies have found that autoimmune gastritis is accompanied by changes in autophagy. The study found that IFN-γ, as a key molecule in the pathogenesis of autoimmune gastritis, activates the transcription of Beclin-1 and upregulates the expression level of LC3-II, thereby activating autophagy and inducing T cell apoptosis (Tu et al. 2011). IFN-γ initiates the autophagy process of gastric epithelial cells by inducing the formation of the autophagosome. Moreover, treatment of mice with specific expression of IFN-γ in gastric epithelial cells using the autophagy inhibitor chloroquine blocked cell lysosomal turnover.

In summary, the changes in autophagy accompanying the development of chronic atrophic gastritis, but the specific mechanism needs further study.

38.2.1.2 The Role of Autophagy in the Progression of Chronic Atrophic Gastritis

In 1988, Correa proposed a classic model of natural progression of intestinal type gastric cancer, namely, "normal mucosa—chronic non-atrophic gastritis—chronic atrophic gastritis—intestinal metaplasia—dysplasia—gastric cancer". Chronic atrophic gastritis is a critical stage in which gastritis is benign or malignant. Chronic atrophic gastritis gradually progresses to intestinal metaplasia, dysplasia, and gastric cancer. Therefore, chronic atrophic gastritis is recognized as a precancerous lesion of gastric cancer. Studies have found that about 4.41% of chronic atrophic gastritis progressed to gastric cancer after 8 years of follow-up. Studies have found that autophagy plays an important role in the progression of chronic atrophic gastritis.

(1) Autophagy and precancerous lesions (intestinal metaplasia and dysplasia)

Intestinal metaplasia and dysplasia are important stages in the progression of gastritis to gastric cancer, marking changes in cell morphology and structure, and gradually embodying "malignant" biological characteristics. Recent studies have found that autophagy plays an important role in the evolution of this state (Li et al. 2018). The study used MNNG reagent to induce precancerous lesions (intestinal metaplasia and heterosexual hyperplasia) in mice. The researchers found that autophagy-related factors ATG5, ATG12, Ambra1, Beclin1, LC3, and p62 were significantly upregulated during modeling (Cai et al. 2018). The researchers found that the traditional Chinese medicine "Astragaloside IV" has a significant therapeutic effect on precancerous lesions in mice, and it has been observed that the expression of autophagy-related genes in mice is gradually downregulated with the improvement of precancerous lesions. The results of this study clearly suggest that autophagy plays an important role in the progression of chronic atrophic gastritis to intestinal metaplasia or heterosexual hyperplasia, but the specific mechanism needs further study.

(2) Autophagy and gastric cancer

① There are many reports that the expression of autophagy-related genes in gastric cancer tissues is significantly upregulated: studies have used immunohistochemistry to detect the expression of LC3A in 188 patients with gastric cancer and adjacent tissues. The results suggest that LC3A is significantly expressed in gastric cancer tissues. Increased and high expression of LC3A is associated with increased risk of recurrence after radical resection of gastric cancer, and is also associated with decreased survival of gastric cancer. The above studies suggest that autophagy is activated during the development of gastric cancer. ② Autophagy can promote the progression of gastric cancer (growth and metastasis): autophagy can protect tumor cells from damage by exercising normal autophagy. Autophagy separates mitochondria and other organelles in cells, which can effectively prevent the spread of the apoptotic factor in the cell and help the tumor cell escape the threat of apoptosis. Studies have confirmed that the use of the anticancer agent MHY218 in gastric cancer can inhibit the progression of gastric cancer by inhibiting autophagy. Another study reported that autophagy promotes gastric cancer progression by activating

AKT phosphorylation and inhibiting PI3K-AKT signaling pathway in human gastric cancer cell lines. Meanwhile, some studies have found that the upregulation of ATG5 with the emergence of drug resistance, the formation of residual cancer stem cell resistance, often lead to reduced sensitivity to certain drugs, and cause tumor recurrence and even metastasis. ③ However, autophagy also can inhibit the formation of early tumors in gastric cancer: in general, autophagy plays a role in inhibiting the development of cancer by limiting inflammatory reactions, tissue destruction, and genomic instability. When autophagy is activated, it inhibits the growth of gastric cancer cells and induces apoptosis. Studies have shown that NF-κB-specific inhibitors can effectively promote the death of gastric cancer cells by upregulating the expression of LC3 and Beclin1, so the anti-inhibition between NF-κB inhibitor, autophagy activation, and apoptosis may become a new direction for the diagnosis and treatment of gastric cancer.

38.2.2 Autophagy in Chronic Non-atrophic Gastritis and Special Gastritis

38.2.2.1 Autophagy and Chronic Non-atrophic Gastritis

According to the consensus opinions on chronic gastritis in China, chronic non-atrophic gastritis (CNAG) is the most common type of chronic gastritis diagnosed by upper gastrointestinal endoscopy in China, accounting for 49.4% of all types of chronic gastritis. The pathological features of CNAG include chronic non-atrophic inflammatory lesions in the gastric mucosa, which are mainly infiltrated by lymphocytes and plasma cells in the gastric mucosa.

H. pylori (Hp) infection is the main cause of CNAG. In fact, CNAG can be regarded as an infectious disease to some extent. It is currently believed that autophagy plays an important role in Hp infection as well as gastric mucosal epithelial injury and repairing (see Sect. 3 of the current chapter for details).

Non-steroidal anti-inflammatory drugs (NSAIDs)-related gastric mucosa injury is also the common factor of CNAG. According to the classical theory, NSAIDs reduce the biosynthesis of prostaglandin (PG) of gastric mucosa by reducing the activity of cyclooxygenase (Cox), leading to gastric epithelial injury. However, recent studies have shown that autophagy-related gastrointestinal epithelial cell death may be an important way of NSAIDs-related mucosa injury. Lee et al. found that the autophagy protein 5 (ATG5) level in gastric mucosa was significantly upregulated after 6 h of indomethacin treatment. Within 24 h, the expression of autophagy markers such as microtubule-associated protein light chain 3B-II (LC3B-II) and Beclin-1 was also significantly increased, which led to autophagy and death of gastric epithelial cells. In addition, Smad-7 plays a protective role in indomethacin-induced gastric epithelial autophagy. Overexpression of Smad-7 inhibits autophagy and the expression of LC3B-II by activating the p38-MAPK signaling pathway. Moreover, Lee et al. also

found that indomethacin-induced gastric mucosa injury was significantly alleviated after the treatment of autophagy inhibitor chloroquine. Another study demonstrates that compared with wild type mice, gastric mucosal epithelial damage of gastrointestinal mucosa epithelial cells-specific *Atg5*-knockout mice was significantly alleviated after indomethacin induction, accompanied by enhanced ERK/Nrf2/HO-1 pathway activity (Harada et al. 2015). These evidences show that autophagy plays a key role in the damage of gastric epithelial cells in CNAG caused by NSAIDs such as indopexin, in which decreased activity of Smad-7 and ERK/Nrf2/Ho-1 pathways may be an important mechanism involved.

38.2.2.2 Autoimmune Gastritis and Autophagy

Autoimmune gastritis is a chronic gastritis caused by autoimmune mechanism. Due to the presence of antibodies against endogenous antigen of gastric tissue, such as anti-internin antibody, anti-parietal cell antibody, anti-gastrin-secreted cell antibody, the corresponding tissue damage or dysfunction can lead to vitamin B12 absorption disorders, gastrin secretion disorders, decreased gastric acid secretion, and so on (New and Thomas 2019).

Autoimmune gastritis is a rare chronic gastritis with low incidence in China. Recent studies have found that IFN-γ can directly attack gastric epithelial cells, playing an important role in the pathogenesis and destruction of autoimmune gastritis. In 3D culture model of gastric somatic cell organs (gastroids culture), the IFN-γ containing supernatant of the immune cells culture medium could lead to the death of gastroids by its cytotoxicity. Therefore, the damage of gastric epithelial cells in autoimmune gastritis is closely related to IFN-γ. Recently, some scholars have further found that IFN-γ could trans-activate the transcription of Beclin-1 and upregulate the expression level of LC3B-II, thus to initiate the autophagy process of gastric epithelial cells by increased formation of autophagosomes. Moreover, the lysosomal turnover of gastric epithelial cells-specific IFN-γ overexpression mice was blocked when treated with the autophagy inhibitor chloroquine. These results suggest that IFN-γ-mediated autophagy may be an important pathogenesis of autoimmune gastritis, but direct evidence is still lacking. The relationship between autoimmune gastritis and autophagy remains to be further explored.

38.2.2.3 Special Types of Chronic Gastritis and Autophagy

In the Kyoto classification of gastritis, the special types of gastritis mainly include Ménétrier disease, allergic gastritis, lymphocytic gastritis, and eosinophilic gastritis. The mucosal lesions caused by these types of chronic gastritis are often associated with immune cell activation and inflammatory cytokine attack. Although there is no literature that directly aim to the role of autophagy in the occurrence and development of the above special types of gastritis, as an important mechanism that mediate and regulate inflammatory responses, autophagy may still participate in the damage process of gastric mucosa and submucosa by specific or non-specific manner.

For example, lymphocytic gastritis is closely related to Hp infection. Its pathological feature is the lymphocytes accumulation within gastric mucosa epithelium and pit cells, which is related to the high concentration of CXCL or CCL family chemokines in this area. The autophagy-dependent secretion may be the crucial mechanism for the enrichment of chemokines in these immune cells. The immune cell chemotaxis mechanism may also exist in allergic gastritis and eosinophilic gastritis that are associated with immune cell aggregation. Moreover, the pathogenesis of Ménétrier disease is closely related to cytomegalovirus infection or TGF-α secretion. Studies have shown that TGF-α may promote autophagy and regulate apoptosis by increasing LC-3 II/LC-3 I ratio. However, the above hypothesis still needs to be verified through in-depth studies in specific animal models or patient tissues.

In conclusion, the studies on the mechanism of autophagy in chronic non-atrophic gastritis and special gastritis are scarce. For these gastritis is closely related to exogenous factors such as Hp infection and cytomegalovirus infection, as well as endogenous factors such as immune cell aggregation and activation, it is very important to study the mechanism of autophagy in this field. The elucidation of the pathogenesis of the special type of gastritis is also helpful to change the current hormone-based clinical treatment, so as to truly benefit the patients.

38.2.3 Role of Autophagy in the Pathogenesis of Inflammatory Bowel Disease

38.2.3.1 Introduction

Inflammatory bowel disease (IBD) results from a complex series of interactions between susceptibility genes, the environment, and the immune system, including ulcerative colon (ulcerative colitis, UC) and Crohn's disease (Crohn's diseases, CD) and undifferentiated colitis (intermediate colitis, IC). Recent studies have shown that the pathogenesis of IBD is closely related to environment, genetics, immunity, microbial infection, and other factors, which leads to immune abnormalities, intestinal flora disorders, oxidative stress effects, and ultimately the occurrence and development of IBD (Baumgart and Sandborn 2012). Current studies suggest that the pathogenesis of IBD may be related to abnormal autophagy in addition to excessive inflammation, changes in immune response, and imbalance of intestinal flora. With the development and application of genome wide association studies (GWAS), more than 160 genetic loci associated with the pathogenesis of IBD have been identified. Single-nucleotide polymorphisms (SNP) of various autophagy-related genes have been found to be related to the sensibility of IBD, making people begin to pay attention to the relationship between autophagy and IBD.

38.2.3.2 IBD-Relevant Autophagy Genes

Studies have found that the pathogenesis of IBD is closely related to gene polymorphism, such as Crohn's disease susceptibility genes NOD2(CARD15), ATG16L1, IR-GM and IL-23R. ATG16L1 and IRGM are autophagy-related proteins, which are closely related to the occurrence and development of IBD.

(1) ATG16L1: ATG16L1 is a key protein in the process of autophagosome formation, located on human chromosome 2q37.1, which plays an important role in the immune response induced by intracellular pathogens and is mainly expressed in intestinal epithelial cells and lymphocytes of the small intestine and colon. ATG16L1 can interact with ATG12 and ATG5 to form atg12-atg5-atg16l1 conjugated complex, and recruit microtubule-associated protein 1 light chain 3 (microtubule-associated protein 1 light chain 3), which can be connected with phosphatidyl ethanolamine and contribute to the extension of autophagy membrane and the formation of autophagosome. The single-nucleotide polymorphism (SNP) sites of ATG16L1 gene are strongly associated with CD, and are important risk factors of CD pathogenesis. Mice with ATG16L1 deficiency showed more sensitization to colitis induced by sodium glucan sulfate (DSS), and showed significantly more leukin (IL-1 and IL-18) than the control group.

The effects of ATG16L1 genetic polymorphism on CD are mainly reflected in the abnormal cell morphology and secretion of Paneth, the defective clearance of intracellular pathogens by intestinal epithelial cells (IEC), and the promotion of inflammation. Paneth cells are characteristic cells of the small intestine, secreting antibacterial substances such as defensin and antibacterial peptide, which play an important role in the innate immune process and inflammatory response of the mucosa. Studies have found that when the expression of ATG16L1 gene in mice is inhibited, the number of particles in Paneth cells in the intestinal tract decreases and the morphology is abnormal. Similarly, Paneth cells of CD patients with ATG16L1 gene mutation also have similar changes. All the above studies indicate that ATG16L1 has a direct impact on the function of Paneth cells.

Saitoh et al. studied the mechanism of ATG16L1 in the regulation of inflammation, and found that ATG16L1 can regulate the activation of endotoxin-mediated inflammatory complexes through the autophagy process, and further regulate the production of proinflammatory cytokines interleukin-1 and il-18. Nguyen et al. studied the regulatory mechanism of ATG16L1. Under physiological conditions, upregulation of mir-30c, mir-130a, mir-106b, and mir-93 can reduce the level of ATG16L1 and inhibit autophagy, thereby blocking autophagy-dependent intracellular bacterial clearance. Lassen et al. found that the inflammatory environment of CD induces cell stress and apoptotic protease activation, which in turn increases caspase-3-mediated ATG16L1 lysis, reduces ATG16L1 level and leads to autophagy abnormalities. ATG16L1 not only plays a role in the process of autophagy, but also plays an important role in other related pathways, which is expected to be a new target for the future treatment of CD.

(2) NOD2/CARD15: NOD2, the first CD-related susceptibility gene, belongs to the NOD like receptor family and is named as CARD15. It is an important pattern recognition receptor that can recognize pathogen-related molecular patterns and thus play a role in innate immunity. NOD2 recognizes the cell wall of bacteria acyl dipeptide, induction of inflammatory corpuscle NLRP 3 formation, activation of MAPK, downstream the nf-kappa B pathway, then raise structure through own caspase activation domain (caspase-activating and recruitment domain, CARD) recruitment and activation of caspase 1, release of inflammatory factors such as IL-1β and IL-18, further causing an immune reaction. NOD2 plays an important role in the autophagy clearance of bacteria, but this process requires the participation of ATG16L1, which is manifested in the interaction between NOD2 and the WD40 domain of ATG16L1 through the CARD domain, thus inducing the autophagy process of cells against the invading pathogen. After NOD2 recognizes intracellular bacteria, ATG16L1 can be collected to the entrance of bacteria in the plasma membrane, while mutation NOD2 cannot complete the above process, resulting in impaired autophagy clearance of bacteria. Sorbara et al. found that ATG16L1 knockout can promote NOD2-induced proinflammatory cytokines production through autophagy-independent pathway, suggesting a close relationship between ATG16L1 and NOD2.

In European and American populations, the common SNP loci of NOD2/CARD15 gene currently mainly include rs2066844 (R702W), rs2066845 (G908R), rs41450053 (L1007fsC), etc. Seidere study found that the risk of CD of the mutant homozygous NOD2 is 20–40 times higher than that of normal people, and the onset age is younger, and the clinical manifestations are more serious, and the probability of ileal stenosis and the need for surgical intervention is significantly increased. However, the above SNP loci were not found to be significantly associated with IBD in Asian and southern African countries, and the incidence of IBD in these countries was much lower than that in northern European and North American countries, which may also confirm the promoting effect of abnormal NOD2/CARD15 gene on the incidence of IBD.

(3) IRGM: IRGM gene is located on human chromosome 5q33.1, and its encoded protein belongs to the immune-related GTPase family, which is expressed in a variety of human cells, and is related to the processes of allogeneic autophagy, proinflammatory cytokine production and apoptosis, etc., playing an important role in the body's immunity. McCarroll showed that the polymorphism of IRGM gene was closely related to CD through GWAS analysis, such as rs10065172 and rs13361189. Liu et al. found that the intestinal Paneth cells of IRGM knockout mice had abnormal localization and morphological changes of secretory particles, and such mice were more susceptible to dss-induced colitis. The above studies suggest that IRGM variation may affect the formation of autophagosomes and the function and morphology of Paneth cells, thereby promoting the occurrence of intestinal inflammation. Studies have found that cd-related SNP can be found at the missing sites upstream of IRGM, and its deletion is correlated with the risk of CD. Subsequent studies have confirmed that mir-196 with

high expression in inflammatory intestinal epithelial cells of patients with CD protects the body by downregulating the expression of IRGM gene. In addition, NOD2 and IRGM interact with each other. NOD2 can promote the oligomerization of IRGM, and promote the interaction between IRGM, and the autophagy regulating molecules ULK1 and BECN1.

38.2.3.3 Pathology and Pathogenesis of IBD

(1) Autophagy and innate immunity

Autophagy is an important part of innate immunity, which can decompose damaged organelles and proteins, decompose pathogens to eliminate them, activate innate immune cells, and regulate innate immune functions. Studies on Paneth cells in the small intestine have shown that Paneth cells can secrete antimicrobial substances to resist intestinal bacterial infection. When the expression of ATG16L1 gene in mice is restricted, the number of particles in Paneth cells decreases and the morphology is abnormal, indicating that ATG16L1 plays an important role in the exocrine process of Paneth cells. Multiple studies have shown that defects in the autophagy process can lead to excessive secretion of IL-1, IL-6, and IL-18. The mechanism is that autophagy-deficient cells produce high levels of reactive oxygen species (ROS) and activate caspase-1, which is dependent on TIR binding protein (Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF), leading to the increase of IL-1 and IL-18. In addition, ATG16L1 knockout mice are more likely to be glucohexaose sodium sulfate (dextran sulfate sodium, DSS)-induced acute colitis, which show the ulcer and lymphocyte infiltration is aggravating, serum inflammatory cytokines IL-1β, IL-6, IL-18. Remission can be achieved by injecting antibodies against IL-1β and IL-18. Intracellular abundance of IL-1β, IL-6, and IL-18 induces apoptosis and inflammatory responses in a range of responses.

(2) Autophagy and adaptive immunity

Autophagy plays an important role in adaptive immune regulation. An important bridge between innate and adaptive immunity is the antigen-presenting cell (APC). In histocompatibility complex-II (MHC-II)-dependent APC, autophagy plays an important role in the process of antigen processing and presentation. ATG16L and IRGM mutations can be involved in the occurrence of CD through the following mechanisms. First, intracellular soluble antigens can be degraded into antigenic peptides by autophagy, recognized by MHC class II molecules and presented to T cells to activate adaptive immunity. Autophagy defects can affect bacterial antigen peptide presentation. Second, the body normally maintains the homeostasis of the immune response by regulating the number of T cells. The number of T cells increased with antigen stimulation and decreased with antigen removal. The sustained activation of Th1, Th2, and Th17 at CD may be related to the autophagy disorder and the decreased ability of the body to regulate the duration and intensity of the adaptive

immune response by controlling the survival time of T cells. Overstabilization of the immune synapse may be one of the mechanisms responsible for the excessive activation of T lymphocytes. Knockout of the expression of ATG16L1 and IRGM in dendritic cells (DCs) causes too close interaction between DCs and T cells, resulting in stable immune synapses and increased activation of T cells, especially Th17 cells. Immune synaptic overstabilization was also observed in DCs isolated from CD patients with ATG16L1 mutation. Adaptive immunity may be one of the reasons for CD susceptibility in ATG16L1 mutation. Third, autophagy defects may reduce the immune tolerance of the body to intestinal symbiotic bacteria and autoantigens, resulting in enhanced intestinal adaptive immunity. Autophagy can not only deal with exogenous antigens, but also remove apoptotic and necrotic autosomal cells, so as to prevent the occurrence of autoimmunity. In addition, autophagy promotes the proliferation and survival of memory T cells by removing excess mitochondria. In the ATG5 deficient mouse model, the number of memory T cells was greatly reduced, and the mice showed enteritis, indicating that autophagy plays an important role in the process of immune homeostasis.

(3) Autophagy and endoplasmic reticulum stress

Endoplasmic reticulum is an important cell that is involved in the folding, modification, transport, and function of intracellular proteins. Under the stimulation of various stress factors, the normal function of endoplasmic reticulum can be affected, resulting in unfolded or misfolded proteins. This process is called endoplasmic reticulum stress. Intestinal endoplasmic reticulum stress can lead to abnormalities in the function of Paneth cells and goblet cells (such as antimicrobial peptides and mucous eggs), and damage the barrier of normal intestinal antimicrobial and mucous proteins, thereby causing continuous inflammatory stimulation and promoting the occurrence of IBD. Autophagy helps to degrade the abnormally folded proteins produced by endoplasmic reticulum and maintain homeostasis. Endoplasmic reticulum stress and NF-kB and TNF-signaling pathways increased in autophagy-deficient mice, leading to spontaneous ileitis similar to CD. These results suggest that autophagy and ER stress may be closely related to the occurrence and development of IBD.

(4) Autophagy and intracellular bacterial infection

Autophagy is one of the immune defense mechanisms of the body to eliminate invading bacteria, and the deficiency of autophagy will affect the cells to remove invading pathogens. In addition to promoting adaptive immune clearance of infection through the mhc-ii presentation pathway, autophagy can also be directly cleared by phagocytosis. Intestinal flora imbalance and persistent bacterial infection may be the cause of IBD. Adherent-invasive Escherichia coli (AIEC) infection was identified in CD patients with ileum involvement. Lapaquette et al. found that autophagy at the physiological level can effectively inhibit the proliferation of AIEC, while there is a large amount of AIEC proliferation in cells with defective IRGM and ATG16L1 genes. Murthy et al. conducted an in-depth study on the mechanism of ATG16L1 mutation leading to bacterial clearance obstacles, and found that caspase3 played an important

role in this process, and the ATG16L1 T300A mutation made it degraded by caspase3. Without activation of caspase3, the autophagy level of the mutant ATG16L1 T300A is not affected. Caspase3 can be activated after the stress state caused by Yersinia enterocolitica infection or the binding of TNF- with the death receptor on the cell membrane, leading to the decomposition of ATG16L1 T300A and the decrease of the autophagy level. The increased secretion of TNF- and il-1 during *E. coli* infection may explain why anti-TNF—therapy is effective in patients with Crohn's disease. The reason may be that TNF—can activate caspase3 to degrade ATG16L1, which instead leads to more TNF—secretion. Studies have shown that AIEC can upregulate the microRNA-30c and microRNA-130a levels of intestinal epithelial cells through the NF-kb pathway, thereby reducing the expression of ATG16L1 and ATG5, inhibiting the autophagy process, and contributing to the survival and further invasion of AIEC. All the appeal studies demonstrated the relationship between autophagy defects and AIEC infection and the pathogenesis of C. Autophagy defects lead to decreased clear ability of intracellular bacteria, persistent intracellular infection, recruitment of more inflammatory cell infiltration, and excessive secretion of cytokines to form chronic granuloma, all of which may be related to the pathogenesis of CD.

(5) Autophagy and abnormal function of Panth cells

Panth cells are a kind of cells located at the base of intestinal gland. As an important part of intestinal antimicrobial biological barrier, they can dissolve the cell wall of intestinal bacteria by secreting bacteriolytic enzymes, defense elements, and other bactericidal substances. Some scholars found that the ability of Panth cells to secrete bactericidal substances in ATG16L1 and ATG5 deficient mice was significantly decreased. In CD patients with ATG16L1 defect, the secretory function of Panth cells is abnormal, which may change the normal intestinal flora by weakening the intestinal antimicrobial biological barrier and cause intestinal injury and chronic inflammation.

(6) Autophagy and intestinal mucosal barrier function

The intestinal mucosal barrier is composed of four parts: mechanical barrier, biological barrier, chemical barrier, and immune barrier, with the function of preventing bacterial and endotoxin migration and pathogenic substances from entering the systemic circulation. Any part of the defect or damage may lead to intestinal homeostasis imbalance, causing intestinal flora migration, enterogenous infection and other diseases. Intestinal mucosal barrier dysfunction is an important basis for the pathogenesis of IBD. Studies have found that autophagy mediates intestinal mucosal barrier injury in IBD patients through multiple pathways, such as regulating the tight connection between intestinal epithelial cells, regulating the expression of inflammatory signals, regulating endoplasmic reticulum stress, and participating in the clearance of pathogenic microorganisms. Nighot et al. found that autophagy could induce the degradation of claudin-2 perforin, thus enhancing the function of intestinal mucosal barrier. Xavier et al. demonstrated that IFN-gamma and other helper T cell type 1 (Th1) cytokines secreted during bacterial infection can induce autophagy, while Th2

cytokines inhibit autophagy. The role of inflammatory cytokines in the pathogenesis of IBD is clear. Autophagy can regulate the secretion of proinflammatory factors and anti-inflammatory factors in both directions, thus participating in the regulation of intestinal mucosal inflammation. Wang's study indicated that ER stress can initiate autophagy in Paneth cells through various pathways. Autophagy defects can lead to excessive activation of endoplasmic reticulum stress in DSS-induced colitis animal models and IBD patients, thus aggravating the severity of IBD (Kaser and Blumberg 2014).

38.2.3.4 Role of Autophagy in IBD and Future Prospects

The pathogenesis of inflammatory bowel disease is complex, and the etiology has not been fully elucidated. Studies have shown that abnormal autophagy may be involved in the pathogenesis of IBD. The regulatory role of autophagy in IBD is also interactive. Under normal conditions, autophagy-related proteins interact with intestinal mucosal immunity and mucosal barrier to jointly maintain intracellular homeostasis. When cells are stimulated by the outside world, the intracellular equilibrium state is broken, and a series of signaling factors are stimulated to regulate autophagy or endogenous protective substances, so as to exert endogenous regulation, promote metabolism, and jointly maintain the survival of cells. When the external stimulation is too strong, the dynamic balance of cells is broken, leading to the occurrence of disease. How to reasonably and effectively induce or inhibit autophagy to maintain or repair the function of intestinal mucosal barrier, protect the integrity of intestinal mucosal barrier, maintain intestinal homeostasis, and thus prevent and control IBD still needs further research.

38.3 Autophagy and Gastrointestinal Infections

38.3.1 Autophagy and Gastrointestinal Bacterial Infectious Diseases

38.3.1.1 Autophagy and Chronic Infection of *H. pylori*

H. pylori is a kind of engraftment in the stomach and duodenum gram-negative, micro-aerobic bacteria can secrete a variety of virulence factors such as urease and lipopolysaccharide, adhesion factor, cytotoxin genes (CagA), a protein cavitating toxin (VacA), such as peptic ulcer, chronic gastritis, the stomach with people closely related diseases such as malignant tumor. At least 75% of gastric cancer is due to *H. pylori* infection, so *H. pylori* is classified as class I carcinogen. Some studies have found that *H. pylori* can re-enter the extracellular environment after completely eliminating extracellular bacteria with gentamicin, suggesting that *H. pylori* is not

only an extracellular pathogen, but also a facultative intracellular bacteria. It can survive not only in epithelial cells, but also in immune cells. *H. pylori's* sojourn in host cells not only increases its resistance to antibiotics, but also enables it to escape the attack of humoral immunity, which may be closely related to the continuous infection of *H. pylori* in the body.

(1) *H. pylori* can induce autophagy

VacA is one of the important substances that induces autophagy. Autophagy was induced by co-culture of purified VacA toxin with AGS cells. On the other hand, autophagy degrades VacA and reduces the toxicity of *H. pylori*. HP0175 is a kind of peptide-based proline isomerase of *H. pylori*. The study of Halder et al. (Halder et al. 2015) found that compared with the cells treated by the wild strain, AGS infected by the mutant strain that eliminated HP0175 showed decreased autophagy, suggesting that HP0175 can upregulate the expression of autophagy-related genes. This may play an important role in autophagy mediated by unfolded protein reactions.

(2) *H. pylori* can also inhibit the occurrence of autophagy

Tanaka et al. (2017) conducted microarray analysis of autophagy-related genes (ATG) in the gastric mucosa of 266 patients infected with *H. pylori*, and found that 16 genes were upregulated and 9 genes were downregulated. Among them, the level of ATG16L1 mRNA of autophagy core component was significantly downregulated, and it was negatively correlated with the colonization density of *H. pylori* and the atrophy degree of gastric mucosa. It suggests that *H. pylori* infection can provide a good living environment for *H. pylori* colonization and promote the cytotoxicity of *H. pylori* by inhibiting autophagy. Microtubule-associated protein 1 light chain 3 (MAP1LC3A) is the main regulator of autophagosome formation. Muhammad et al. found that MAP1LC3A variant strain 1 (MAP1LC3Av1) was methylated and silenced in the gastric cancer tissues infected by *H. pylori* and adjacent non-cancerous tissues, but not in the gastric tissues infected by *H. pylori*. In addition, in vitro studies, MAP1LC3Av knockdown cells showed stronger proliferation and invasiveness. All the above evidences suggest that the inactivation of MAP1LC3Av1 destroys the autophagy pathway, which may lead to carcinogenesis of gastric epithelial cells.

38.3.1.2 Autophagy in Intestinal Tuberculosis

Intestinal tuberculosis (ITB) is a chronic intestinal-specific infection caused by Mycobacterium tuberculosis (MTB). Once occurs, often leads to abdominal pain, diarrhea, hematochezia, and other non-specific gastrointestinal clinical manifestations. Macrophages are the main target cells of MTB during the occurrence and development of ITB, as well as the main defense cells of the body. Autophagy, as an important immune regulatory mechanism, can participate in the intestinal infection and defense process of MTB from multiple aspects, and is also reversely regulated by MTB.

(1) Mechanism of autophagy promoting MTB clearance

Autophagy can directly promote MTB clearance. It has been found that the use of vitamin D and lipopolysaccharide can induce autophagy of macrophages and enhance the direct clearance of MTB by macrophages. On the one hand, autophagy activation can promote the maturation of MTB phagocytes in macrophages, which is conducive to MTB clearance. On the other hand, autophagy plays a bactericidal role by promoting the secretion of antimicrobial peptides in lysosomes.

(2) Activation of the natural immune system in the progression of intestinal tuberculosis is involved in the occurrence of autophagy

During the occurrence of intestinal tuberculosis, activation of the natural immune system can induce autophagy of macrophages. After the invasion of MTB into the intestinal tract, macrophages recruit and activate the infected area, and recognize the pathogen-associated molecular patterns (PAMP) of MTB through the pattern recognition receptor (PRR) on its surface, thus activating autophagy and initiating immune defense. Among them, toll-like receptor (TLRs), c-type lectin receptor, scavenger receptor, NOD1 and NOD2 receptor in the PRR family of macrophages are all involved in the recognition of MTB by macrophages, and then initiate autophagy to resist the infection of MTB. Studies have found that TLR4 on the surface of macrophages can recognize lipids, glycoproteins, and secreted proteins in the cell wall of MTB, activate downstream signaling pathways, and promote beclin-1 to form initiation complexes with PI3KC3 and PI3P, thus forming autophagy initiation reactions. PI3KC3 complex plays an important role in the formation and maturation of autophagosomes and promotes the clearance of MTB. Meanwhile, activation of TLR4-related signaling pathway can regulate the stability of autophagy-related signaling molecules beclin-1 and ULK1 through TRAF6, thereby regulating autophagy. Other studies have found that in macrophages infected with MTB, NOD2 receptors on the surface of macrophages can activate downstream signaling pathways by recognizing bacterial MDP, and produce a large number of inflammatory factors such as il-1b and tnf-a, inducing the expression of autophagy markers such as LC3, thereby promoting the occurrence of autophagy (Shaw et al. 2011).

(3) MTB can also reverse regulate the occurrence of autophagy

MTB infection can inhibit autophagy by inducing activation of mTOR signaling pathway. In addition, MTB can express autophagy inhibiting factors such as MTB Eis protein, which can specifically regulate the activity of JNK and inhibit the activation of beclin-1, thus inhibiting the occurrence of autophagy. In addition, at the early stage of MTB infection, antigenic target proteins can be secreted to block the maturation of phagocytes and their fusion with autophagic lysosomes.

38.3.2 Autophagy and Enterovirus Infectious Diseases

Autophagy plays an important role in the process of virus infection. Autophagy can eliminate viruses by degrading viruses, presenting viral antigens and activating the immune response. Meanwhile, viruses can also evade autophagy and maintain their own survival and replication. RNA viruses, in particular, can rapidly alter the genome and evolve, thus inhibiting the autophagy of host cells, which is conducive to the replication of viruses themselves.

Human enteroviruses belong to the enterovirus genus of the small ribonucleic acid viridae family, including poliovirus, coxsackievirus, Ecovirus, and new enteroviruses. There are more than 100 kinds or subspecies of viruses. Poliovirus, Ecovirus 7, Coxsackievirus A16, Coxsackievirus B3, Coxsackievirus B4, and enterovirus 71 infections have been shown to promote autophagy formation, and autophagy promotes viral self-replication and protein expression.

38.3.2.1 Viral Infection and Cell Autophagy

(1) The mechanism of autophagy induced by virus

Virus is an intracellular infectious microorganism, and autophagy is an effective process to maintain cell homeostasis. Several events in viral replication, such as receptor interaction and endoplasmic reticulum stress, can trigger downstream autophagy. It has been found that GTPase family M protein (IRGM) is a common target protein of five RNA viruses, such as retroviruses, Flaviviridae, paramyxoviruses, orthomyxoviruses, and tunica viruses, which interact with autophagy-related proteins. When the expression of IRGM was inhibited, autophages induced by virus particles such as measles virus, human immunodeficiency virus type 1, and hepatitis C virus decreased significantly.

① Endoplasmic reticulum stress is involved in the activation of autophagy pathway. Endoplasmic reticulum stress can prevent proteins from entering the endoplasmic reticulum by processing misfolded proteins in the endoplasmic reticulum through unfolded protein response (UPR). UPR is the main protective and compensatory mechanism of endoplasmic reticulum stress. Unfolded protein response has three signaling pathways: protein kinase-like endoplasmic reticulum kinase (PERK), endoplasmic reticulum transmembrane protein inositol-requiring enzyme 1 (IRE1) and transcription activator 6 (ATF6). When the virus is infected, a large number of viral proteins are synthesized in the cells, which increases the burden of endoplasmic reticulum, and then increases the accumulation of unfolded proteins and misfolded proteins to cause unfolded protein reaction, thus activating endoplasmic reticulum stress in host cells and inducing autophagy.

② Receptor interaction Virus binding to cell surface receptor can directly induce autophagy and initiate cell anti-infection mechanism. CD46 receptors are ubiquitous on the cell surface and can bind to a variety of viruses to induce autophagy.

CD46 can be divided into two domains, Cyt-1 and Cyt-2, according to the carboxyl end. CD46-Cyt-1 can induce autophagy by interacting with the skeleton protein GOPC and coupling with the autophagy initiation complex Vps-34/Beclin. It has been reported that measles virus can induce autophagy through CD46-Cyt-1/GOPC pathway by binding to CD46 molecule on cell membrane. Human immunodeficiency virus type 1 envelope glycoprotein gp120 and cardiomyocyte *N*-methyl-D-aspartate receptor (NMDA) induce autophagy, which involves c-JUN amino-terminal kinase (JNK) and PI3K.

(2) Interaction between Virus and Cell Autophagy

① The antiviral effect of autophagy: Autophagy is the process of maintaining homeostasis in cells. Viral infection can lead to the disorder of intracellular environment. When the virus is infected, the body will activate the autophagy mechanism to resist the virus infection. Autophagy can activate innate immunity by transporting viral nucleic acid to intracellular receptors and presenting viral antigens to MHC-I and MHC-II molecules to activate adaptive immune response. At the same time, autophages can transfer viruses from cytoplasm to lysosomes, fuse with lysosomes, phagocytize and degrade viruses.

② Cell autophagy promotes virus infection: Autophagy is an inherent metabolic process in eukaryotic cells. However, in the process of virus infection and evolution, viruses may adapt to cell autophagy through some mechanisms, which is conducive to their own replication. Viruses may adapt to autophagy in the following ways: (1) Autophages can be the place where viruses replicate. The formation of viral replication sites often leads to membrane rearrangement and cytoskeleton remodeling. Similar rearrangements occur during the formation of aggregates and autophages in cells to promote protein degradation. Autophagy provides an assembly platform for the replication complexes of positive-stranded RNA viruses; (2) Autophagy is beneficial to the replication and expression of viral genes. Non-structural protein 4 (NSP4) of rotavirus releases calcium ions from endoplasmic reticulum into cytoplasm, activates calcium ion/calmodulin kinase-β and 5′-adenosine monophosphate-activated protein kinase (AMPK) signaling pathways, thus initiating autophagy and transporting viral proteins from endoplasmic reticulum to viral replication through autophagic membrane transport process, causing rotavirus infection.

38.3.2.2 Human Enterovirus and Autophagy

(1) Coxsackievirus and Autophagy

When coxsackievirus B3 (CB3) infects Hela and HEK293T cells, the expression of GFP-LC3 increases, and the proportion of LC3-II/LC3-I increases, suggesting that

CB3 infection induces autophagy formation. However, autophagy-mediated protein degradation marker p62 did not change significantly after CB3 infection, suggesting that CB3 infection did not promote protein degradation in lysosomes. Autophagy promotes viral replication in CB3 infection. Rapamycin and starvation enhanced CB3 replication, while CB3 replication products decreased when 3-MA or RNA interfered with the expression of Beclin 1, Vps34, and Atg7. BPIFP3, a novel autophagy regulator, has been reported to inhibit CB3 replication by inhibiting key links in the autophagy process. When BPIFP3 is absent, CB3 replication is greatly enhanced. Receptor-Interacting Protein Kinase-3 (RIP3) is a necrosis regulator, which promotes autophagy formation during CB3 infection. In the late stage of CB3 infection, RIP3 is cleaved by CB3-encoded cysteine protease 3c, eliminating RIP3-mediated necrosis signal, inducing non-necrotic cell death, inhibiting cell necrosis and benefiting CB3 from autophagy regulation.

Infection of primary rat neurons with Coxsackievirus B4 (CB4) induces autophagy and LC3-II accumulation; 3-MA inhibits autophagy by inhibiting the activation of calpain, thus reducing CB4 replication. Coxsackievirus A16 (CA16) infection has been shown to promote autophagy formation and enhance self-replication through autophagy (Song et al. 2018). The expression of CA16 non-structural virus protein 2C can enhance the activation of IRGM promoter and induce autophagy. In addition, CA16 infection can inhibit the AKT/mTOR signaling pathway that negatively regulates the formation of autophages, and activate the extracellular regulated protein kinase (ERK) signaling pathway to induce autophagy.

(2) Poliovirus and Autophagy

Poliovirus (PV) induces the formation of intracellular vesicles at the early stage of infection and bilayer membrane vesicles at the late stage of infection. These bilayer vesicles and autophages exhibit many similar characteristics, including the division of bilayer membranes enclosing the intracytoplasmic cavity, the acquisition of intracellular labeled lysosome-associated membrane protein-1 (LAMP-1) and the aggregation of host protein LC3. The GFP-LC3 construct was used to study the vesicles induced by PV. The results showed that the GFP-LC3 signal was co-localized with the PV3A protein and the PV double-stranded RNA replication intermediates. At the early stage of PV replication, when PV2BC and 3A proteins were co-expressed in 293T cells of human embryonic kidney (HEK), the co-localization of autophagy marker GFP-LC3 and LAMP-1 indicated that PV infection-induced autophagy maturation. When autophages are transported to lysosomes, vesicles acidify, and PV proliferates vigorously in mature acidic vesicles, and vesicle acidification can promote the maturation of infectious PV particles.

(3) Ecovirus 7 and autophagy

When E7 enters Caco-2 polarized intestinal epithelial cells through endocytosis, autophagy-related proteins are required. Silencing autophagy-related genes such as Atg12, Atg14, Atg16, Beclin1, and LC3 had no effect on the adsorption of virus on cell surface, but could affect the upstream mechanism of E7 dehulling, suggesting that autophagy played an important role in the viral penetration stage.

(4) Enterovirus 71 and autophagy

Enterovirus 71 (EV71) infection promotes the occurrence of autophagy. With the prolongation of infection time and the increase of infection dose, the level of autophagy gradually increases. Autophagy inducer rapamycin enhanced EV71 replication, while autophagy inhibitor 3-MA inhibited EV71 replication. Non-structural protein 2BC of EV71 can trigger autophagic lysosome formation and facilitate virus replication. After blocking autophagic lysosome production with chloroquine, the titer, copy, and protein of EV71 decreased. It has been reported that Beclin1, Vps34, NGLY1, and VCP, which are beneficial to autophagy formation, promote EV71 replication. EV71 can induce multiple autophagy steps to complete its own replication.

(5) Human cytomegalovirus and autophagy

Human cytomegalovirus (HCMV) belongs to herpes virus and has a high infection rate in the population. About 90% of the population showed HCMV positive reaction. Although HCMV infection in healthy individuals is usually asymptomatic, for individuals with immunodeficiency, HCMV is the main cause of disease and death. At the same time, HCMV has the characteristics of latency-activation of herpes virus, and can exist in host cells for a long time. HCMV can evade the antiviral mechanism of autophagy by expressing a variety of specific anti-autophagic proteins. However, in order to replicate, proliferate, and establish latent infection, HCMV can also use autophagy instead of just fighting it. Autophagy can be used wholly or partially by HCMV, which can optimize the transmission or persistence of the virus.

(6) Summary and Prospect

Autophagy is a common programmed cell death mechanism in eukaryotic cells. Viruses, as a specific intracellular parasite, must interact with autophagy in the course of infection. In the process of virus infection, the interaction between autophagy and virus may be that autophagy successfully prevents virus replication, or that virus uses or inhibits autophagy of host cells to serve its own replication. At present, antiviral drugs mainly act on viral proteins. Because enteroviruses are easy to mutate and recombine, there are no effective therapeutic drugs. Understanding the role of autophagy in enterovirus infection can develop new fields for the treatment of enteroviruses.

38.4 Autophagy and Intestinal Microecology

38.4.1 The Role of Autophagy in Maintaining Intestinal Microbiota Homeostasis

Autophagy, a self-eating process, is an important mechanism for cells to maintain material turnover. When aging proteins, damaged organelles, and other wastes occur

in the cell, autophagic vesicles will wrap them up and send them to lysosomes for degradation and recycling, so as to ensure the metabolism of the cell itself and the renewal of some organelles. Intestinal autophagy plays an important role in regulating the diversity and composition of intestinal microbiota.

The autophagy process relies on proteins encoded by autophagy-related genes (Atg). So far, more than 40 autophagy-related genes have been found. Autophagy related genes are crucial for the formation of autophagosomes and the promotion of cell survival. Multiple ATGs play important roles in intestinal mucosal barrier function and intestinal flora homeostasis. ATG16L1, ATG5, and ATG7 play an important role in maintaining the morphology of pan cells and secreting antimicrobial peptides regulating the intestinal microenvironment. When ATG16L1, ATG5, and ATG7 are defective, they will lead to the destruction of the structure of mouse pantoli cells, the disorder of secretion, and the abnormality of granulosa exocytosis pathway, resulting in the peroxisomal value-added activation of receptor gene, which will directly damage the intestinal mucosal barrier function and make intestinal flora translocation. Studies have reported that the absence of intestinal epithelial Atg5 leads to significant changes in the intestinal microbiota of mice, resulting in a decrease in the diversity, as indicated by a decrease in the number of Akk bacteria, rumen coccaceae, and spirospiaceae, while an increase in the number of proinflammatory bacteria (candidatus athromitus) and potential pathogens (pasteuriaceae). Autophagy genes ATG16L1 and phosphatidyl ethanolamine (PE) and type of ubiquitin molecules LC3 connection, affect autophagy body form and function of the key step, by building contains ATG16L1 and allele (ATG16L1HM) in mice, and reduce ATG16L1 expression decreased and autophagy, found that mice intestinal ZhongGe gram-negative bacteria rat citric acid bacillus (intestinal pathogenic bacteria) decreased, thus protecting intestinal damage (Oh and Lee 2014).

38.4.2 Autophagy Mediated by Microbe

The primary function of the autophagy pathway is to adapt to nutrient deprivation by reusing energy and small molecules. However, recent studies have found that autophagy plays an important role in the innate antibacterial immune defense of the intestinal tract of eukaryotes (Girardin et al. 2003). In the event of pathogen invasion, autophagy can directly remove microorganisms in cells by targeting phagocytic microorganism degradation lysosomes. Intestinal flora can also promote autophagy in the body by producing polyamines and vitamin B6.

38.4.2.1 Microbe-Mediated Autophagy Initiation Signaling Activation

When intracellular bacteria destroy cell membranes and cause acute amino acid deficiency, some cell membrane receptors or cytoplasmic receptors are activated. More

importantly, the downstream signaling pathway of these receptors can activate multiple stages of autophagy, such as phagocytic vesicle nucleation, cargo loading and phagocytic vesicle extension. Studies have shown that group A streptococcus and measles virus can activate autophagy through CD46/GOPC/Beclin1 signaling pathway. In addition, the intestinal macrophages showed toll-like receptors (TLRs) that can identify specific microorganisms on the surface of the intestinal flora associated molecular patterns (MAMPs) (including the lipopolysaccharide (LPS) and teichoic acid (LTA) and peptidoglycan (PGN), mannose, bacterial DNA, glucan, etc.) and its downstream MyD88 and TRIF via combined with autophagy genes Beclin1 mutually competitive and reduce the combination of the Bcl—2 and Beclin1, so as to activate autophagy (Xu et al. 2007).

38.4.2.2 Microbe Activates the Autophagic Vacuole

Cytosolic nod-like receptor (NLRs) is a kind of protein family that can recognize intestinal bacterial peptidoglycan. Nod1 and Nod2 belong to the NLRs family. It has been reported that Nod1 and Nod2 can activate the autophagy defense system by binding to atg-5-atg12-atg16l1 complex during autophagy phagocytic vesicle elongation.

38.4.2.3 Microbe Activates the Fusion of Autophagosome and Lysosome

DNA damage-regulated autophagy modulator protein 1 (Dram1) is a lysosomal protein co-located with autophagosome. The number of intestinal mycobacteria was regulated by Dram1 protein. Other studies have confirmed that Dram1 mediates p62-dependent selective antimicrobial autophagy downstream of the tlr-myd88-nf-b signaling pathway. Dram1 was required for autophagosome formation and fusion of autophagosome and lysosome regulated by mycobacterium. Dram1 has been shown to play an important role in autophagosome maturation in response to intracellular pathogen invasion.

Whether bacterial infection can activate the mitochondrial autophagy pathway, the researchers tried a variety of bacteria, including listeria, salmonella, escherichia coli, and citrobacter, to systematically analyze the occurrence of mitochondrial autophagy. Studies have found that listeria and salmonella have the function of inducing mitochondrial autophagy. Further research on listeria also found that listeria can produce a protein called hemolysin O, which can cause mitochondrial damage in cells and thus induce mitochondrial autophagy.

38.5 Autophagy and Gastrointestinal Motility Disorders

38.5.1 The Relationship Between Cajal Interstitial Cells (ICC) and Gastrointestinal Motility—ICC Plays an Important Role in Maintaining Normal Gastrointestinal Motility and Is an Important Link in Regulating Gastrointestinal Motility

In 1893, Santiago Ramóny Cajal described for the first time a special cell type, which is located between endings of autonomic neurons and muscular cells. They were structurally and functionally further characterized in the gut musculature and named mesenchymal cells of Cajal (ICC). In the last 100 years, studies have focused on ICC were profoundly understood. As the pacemaker activity, ICC was arising from the interstitial cell of gastrointestinal tract. ICCs are known to provide pacemaker activity, propagation pathways for slow waves, transduction of inputs from motor neurons, the contraction of gastrointestinal smooth muscle, and neurotransmission. It is considered that ICC network loss and pathological damage may be related to the many types of gastrointestinal motility disorders. Currently, gastrointestinal motility disorders may be related to the disorders of development and differentiation, abnormal structure, quantity, and distribution of ICC. Because ICCs have unique features that are specific for the gut musculature, they are an ideal target for pharmacological action. Many studies have been performed in recent years that bring us closer to our understanding of the physiological role of ICC in controlling GI motility and their role in pathophysiology of motility disorders.

Different ICC populations were found in the gut muscle coat with region-specific location, region-specific ultrastructural features, and function. ICC was classified according to cell morphology, location, and function: (1) Classification according to cell morphology and location: Myenteric ICC (ICC-MY) is a form of a cellular network around the myenteric plexus in the space between the circular and longitudinal layers of muscle; Submucosal ICC (ICC-SM) distributes along the submucosal layer of the gastrointestinal muscular layer; Deep muscular ICC (ICC-DMP) designates the nerve plexus between the thick outer and thin inner subdivision of the circular muscle layer; Intramuscular ICC (ICC-IM), located in the muscular layer. (2) Classification according to ICC cell function: The network of IC-MY in small intestine and ICC-SMP in colon which is believed to be the origin of electrical slow waves is morphologically independent from but associated with the myenteric plexus. They are involved in regulation of intestinal smooth muscle nerve signals, which includes ICC-IM distributed in the gastrointestinal tract. There are three main functions for ICC that have been proposed: to pace slow waves and regulate their propagation; to mediate enteric neuronal signals to smooth muscle cells; and to act as mechanoreceptors coupled with ICC via gap junctions, and the functional unit thus formed enables rhythmically synchronized contractions and relaxations (Klein et al. 2013).

Studies have confirmed that abnormal ICC structure and loss of ICC lead to gastrointestinal dysmotility in humans and animals, including idiopathic achalasia, diabetic gastrointestinal disease, Hirschsprung's disease, chronic intestinal pseudo-obstruction, anorectal malformation, and slow transit constipation. Research suggests that in the pathogenesis of achalasia, especially in the development of the LES high-pressure zone, depletion of ICC networks and potential changes in the electrical activity of smooth muscle cells may play a crucial role. Research findings have shown that immature ultrastructural features of ICCs in infantile hypertrophic pyloric stenosis. Their findings were supported by studies, which provided substantial evidence that ICCs might have a role in the pathogenesis of hypertrophic pyloric stenosis. Diabetic gastrointestinal disease is one of the common complications of diabetes mellitus. There is increasing evidence for specific cellular changes in the stomach of patients with diabetic (DG) and idiopathic (IG) gastroparesis. The most significant findings are loss of interstitial cells of Cajal (ICC), neuronal abnormalities, and an immune cellular infiltrate. Chronic intestinal pseudo-obstruction (CIIP) is characterized by alteration of the ICC network. Decreased ICC density along with loss of processes and damaged intracellular cytoskeleton and organelles have been reported in patients with CIIP. Several investigators have studied the distribution of ICCs in the ganglionic and aganglionic bowel of patients with Hirschsprung's disease (HD), which described reduced number of ICCs with disrupted network. Recent research findings defects in the population of intestinal pacemaker cells may underlie the colonic hypomotility seen in high anorectal malformations and hence may contribute to refractory constipation. The present study suggests decrease in ICC and in neuronal cells in the slow transit constipation. A decrease in c-kit positive cells was noted in all regions of the sigmoid colon. It seems that ICC plays an important role in generation of the smooth muscle electrical slow wave that determines contractile activity.

38.5.2 Association Between Autophagy and Abnormalities in the Structure and Number of ICC Cells—Excessive Autophagy in ICC May be One of the Key Factors in Gastrointestinal Motility Disorders

Autophagy, also known as type II cell programmed death, is widely existed in eukaryotic cells and is involved in cell growth, development, and pathophysiological processes. Autophagy is not only a ubiquitous normal physiological process, but also one of the cell's defense mechanisms against adverse environment, and it is also a self-protection mechanism of cells. The nature of autophagy is a cup-shaped segmentation membrane derived from the endoplasmic reticulum, Golgi, or endosomal lipid bilayer. With a gradually prolonged structure, the segmentation membrane engulfs damaged organelles and some cytoplasm and forms autophagosomes. Autophagy has dual effect on cells. In normal cells, basal levels of autophagy are maintained at a lower level to keep cell homeostasis, and to ensure normal physiological function

of cells, with a similar effect as scavengers. When homeostasis changes, such as developmental differentiation, aging, or effects induced by external adverse stimuli, autophagy is immediately induced, and excessive autophagy will destructively degrade a large number of useful proteins and organelles, resulting in cells' incapacity to perform normal function, which will lead to cell disruption and programmed cell death. The current gold standard for measuring the level of autophagy in cells is to directly count the number of autophagic vacuoles in the cells using a transmission electron microscope.

The generation of ICC pacing potential and slow-wave propagation is affected by biological activities such as ion environment, neurotransmitters, and hormones, among which Ca^{2+} has the closest relationship. Studies have shown that ICC expresses a low-threshold Ca^{2+} channel, which causes periodic depolarization of ex vivo ICC and is closely related to changes in extracellular Ca^{2+} concentration. Intracellular Ca^{2+} overload is one of the initiation factors of autophagy and is the ultimate common pathway leading to cell damage and death. Because intracellular Ca^{2+} concentration changes are closely related to systolic activity of gastrointestinal smooth muscle, high concentration of Ca^{2+} can cause smooth muscle contraction, while low concentration of Ca^{2+} can cause smooth muscle relaxation. Intracellular Ca^{2+} overload is one of the initiation factors of ICC autophagy. Increased intracellular $Ca2^{+}$ concentration can directly activate some proteolytic enzymes, or act as a third messenger to influence cellular gene expression and induce autophagy. Therefore, excessive autophagy in ICC may be one of the key factors in gastrointestinal motility disorders.

38.5.3 Mechanism of Regulating Gastrointestinal Motility Disorders by Excessive Autophagy in ICC

Studies have shown that excessive autophagy in ICC may be one of the mechanisms in the pathogenesis of gastrointestinal motility disorders. Under the stimulation of the signal, autophagy begins in cells, firstly forming a bilayer membrane vacuole in the cytoplasm wrapping materials to be degraded, such as mitochondria and endoplasmic reticulum fragments, which is called autophagosomes. Autophagy is a complex process involving multiple factors. Among them, Beclin1 gene, also known as BECN1 gene, is a key factor involved in the regulation of autophagy, and is an important condition in the formation of autophagosome. After autophagy is induced, it is first combined with phosphatidylinositol 3-kinase (type III P13K) to form a complex that regulates the localization of other ATG proteins in the autophagy precursor structure, and regulates the synthesis of autophagosome membranes, which is a key gene in the initiation of autophagy. The BECN1 gene also regulates autophagy activity, and the number of the gene represents the activity of autophagy and is an important indicator for evaluating autophagy activity. Microtubule-associated protein 1 light chain 3 (LC3) is a homolog of the yeast ATG8 gene in mammalian cells. LC3A is routinely

expressed and freely present in the cytoplasm. During autophagy, LC3A is processed and modified by ubiquitin-like system to produce LC3B. LC3B is covalently linked to phosphatidylethanolamine (PE) on the surface of autophagy membrane to form liposoluble LC3B-PE, which is involved in the extension of autophagosome membrane, until the formation of autophagolysosome. The LC3B protein binds and is always localized on the membrane of autophagic vacuoles, and its number is directly proportional to the activity and the level of autophagy, and has been used as one of the specific methods for detecting the expression level of autophagy (Mizushima et al. 2010). It is suggested that the autophagy process is mainly regulated by the PI3K-Akt-mTOR signaling pathway and the Beclin 1 complex.

When the level of intracellular autophagy is elevated, excessive autophagy will occur, which may be manifested by an increase in the number of autophagic vacuoles in the cells, an increase in Beclin1 and LC3B proteins, changes in intracellular structure and the cell numbers due to programmed cell deaths. Studies have confirmed that abnormalities in the structure and number of ICC in intestinal motility disorders may be associated with excessive autophagy in ICC. The berberine hydrochloride was used to construct a rat model of slow transit constipation, detected colonic transit time in rats, and observed morphological changes of ICC and c-Kit protein expression in rat intestinal tract by IF technique, and detected ICC-specific proteins c-kit and Beclin1, LC3B with immunoblotting. The results showed that the colonic transit time of rats with slow transit constipation was significantly prolonged and the exercise capacity was significantly reduced, indicating abnormalities of intestinal myoelectric activity; in addition, compared with the normal group, the number of ICC cells in the intestine is significantly reduced, the ICC interaction network structure was obviously thinner, and the expression levels of Beclin1 and LC3B in the intestinal tissues were significantly higher, indicating that autophagy occurred in the ICC. Therefore, autophagy phenomenon occurred in the ICC in the intestinal tract of the slow transit constipation rat model, which may be one of the reasons leading to the decrease in the number of ICC and slow transit constipation. Based on ICC autophagy, the cause of intestinal motility abnormality in colitis mice was induced by glucose sodium sulfate solution, which measured the tension of isolated smooth muscle strips, observed the ultrastructure of ICC cells under high power transmission electron microscope, and detected the expression levels of c-kit, Beclin1, LC3B protein, and mRNA in intestinal tissues. Results showed that, in colitis mice, the contraction amplitude of colon smooth muscle strips decreased, the frequency of contraction increased, which is similar to the kinetic abnormality of intestinal tract in patients with colitis. And the structure of ICC was abnormal compared with the normal group, with excessive expression of Beclin1 and LC3B protein and excessive autophagy in the colon tissue, which leading to programmed cell death, with a decrease in c-kit protein expression, that is, a decrease in the number of ICC.

38.5.4 Relationship Between Autophagy and Gastrointestinal Motility Disorders/Functional Gastrointestinal Disorders

38.5.4.1 Autophagy Mediates the Protective Effect of Epithelial Cells in Eosinophilic Esophagitis (EoE)

Study found that EoE-associated inflammation promotes autophagy and basal cell proliferation in mice EoE and esophageal organelle models (Whelan et al. 2017). Chloroquine enhances basal cell proliferation and inhibits autophagic fluxes of esophageal keratinocytes to stimulate EoE-associated cytokines, including tumor necrosis factor and interleukin-13α, which are characterized by reactive oxygen species-dependent autophagic flux activation. Oxidative stress in EoE mice, esophageal cells, and human esophageal cancer cells can be enhanced by chloroquine treatment or autophagy inhibition by Beclin-1 or ATG-7 depletion. Compared with healthy subjects and patients with EOE remission, active infection and number of autophagic vacuoles are increased in pediatric esophageal epithelial EoE patients.

38.5.4.2 ICC Autophagy and Functional Dyspepsia

ICC was quantified by ICC-specific membrane protein c-kit. The results showed that the number of gastric ICC in FD rats was significantly lower than that in normal rats. There were a large number of autophagic vacuoles with bilayer membrane structure, which is consistent with excessive autophagy in cells, suggesting that excessive autophagy occurs in gastric ICC in FD rats and excessive autophagy in ICC may be one of the mechanisms leading to decreased gastric motility in FD. ICC-MY is distributed between the circular muscle and the longitudinal muscle in gastric antrum, and is mainly involved in gastrointestinal pacing. In the FD rat model, excessive autophagy in ICC and structural disorder and decreased number of ICC-MY were observed, suggesting the presence of excessive autophagy. At the same time, it is suggested that the pathogenesis of functional dyspepsia may be related to increased mRNA expression of Beclin1 protein and LC3B, which leads to increased autophagy activity of ICC and increased expression of autophagic vacuoles.

38.5.4.3 ICC Autophagy and Slow Transit Constipation (STC)

Recent studies have found that ICC autophagy may be associated with the pathogenesis of STC. The Cajal interstitial cells between the longitudinal and circular muscles can produce slow waves that cause automatic rhythmic movement of the gastrointestinal smooth muscle. The slow wave generated by ICC is closely related to the change of intracellular Ca^{2+}. The main cause of increased intracellular Ca^{2+}

is extracellular Ca^{2+} influx and release from calcium store. When Ca^{2+} in the calcium store is depleted, the calcium channel controlling the calcium store is activated, and leads to a large amount of extracellular Ca^{2+} influx and intracellular Ca^{2+} overload, which induces autophagic apoptosis. Intracellular Ca^{2+} overload can induce autophagy, which is one of the initiating factors of autophagy in cells. Therefore, excessive autophagy in ICC may be one of the key factors in the pathogenesis of STC.

38.5.4.4 ICC Autophagy and Diabetic Gastroparesis

At present, autophagy has been widely studied in many complications of diabetes. In a study of insulin resistance in diabetes, it is found that inhibition of JNK pathway can downregulate autophagy, thereby improving insulin resistance in diabetes. It has been found that highly expressed autophagy proteins can be detected in gastric tissues of diabetic rats. Meanwhile, the reduced contractility was found in isolated gastric smooth muscle cells cultured in high glucose, suggesting that high glucose may induce autophagy of gastric smooth muscle cells by activating JNK signaling pathway. Therefore, the abnormal function of autophagy may play an important role in the pathogenesis of diabetic gastroparesis.

38.5.4.5 Regulation of ICC Autophagy—A New Target for the Treatment of Gastrointestinal Motility Disorders

All in all, ICC has become a therapeutic target for gastrointestinal motility disorders, and autophagy regulation is a potential therapeutic measure for many diseases, which can be realized through multilevel and multipath interference. To further study the relationship between gastrointestinal motility disorder and autophagy of ICC, and to explore ways to strengthen this beneficial autophagy by regulating upstream signaling pathway, may be beneficial to the prevention and treatment of gastrointestinal motility disorder diseases.

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Part IX
Autophagy and Respiratory Diseases

The respiratory system consists of the respiratory tract, including the nasal cavity, pharynx, larynx, trachea, bronchi, and lung. Throughout the development of internal medicine, respiratory diseases have always been the main disease affecting the quality of public life and leading to disease death. Many respiratory diseases, including respiratory infections such as tuberculosis, acute lung injury, asthma, chronic obstructive pulmonary disease, pulmonary vascular disease, and pulmonary fibrosis, are still common or chronic diseases that afflict people. Many diseases of the respiratory system have similar pathological changes and symptoms, such as microbial infections, chronic inflammation, tissue remodeling in the lung airways and lung parenchyma, tissue hypoxia, or oxidative stress damage. Although the role and mechanism of autophagy in the pathogenesis of the respiratory system have just been recognized, more and more studies have shown that autophagy dysfunction or disorder are key mechanisms of various respiratory diseases. For example, autophagy affects the course of respiratory diseases by regulation of cell growth, death, inflammatory response, and pathogenic microbial clearance. Therefore, key components that activate or inhibit autophagy may alter the progression of these diseases. It is worth noting that autophagy produces different effects on different respiratory diseases or different stages of the same disease. Thus, autophagy, especially the activation of autophagic flux, determines the development and outcome of many respiratory diseases. Understanding of the role and mechanism of autophagy will provide useful information for drug development of respiratory diseases.

Chapter 39
Chronic Obstructive Pulmonary Disease and Autophagy

Xiaoxi Lv, Ke Li, and Zhuowei Hu

Abstract Chronic Obstructive Pulmonary Disease (COPD) is a classical chronic respiratory disease with the pathological changes involving the bronchi and alveoli. Many of the risk factors of COPD can induce autophagy in different kinds of cells in lung tissue including alveolar epithelial cells, broncho epithelial cells, and fibroblasts. Over-activation of autophagy may cause emphysema by inducing autophagic cell death. However, the bronchitis and fibrosis may be mainly caused by autophagic flux blocking. Thus, understanding the role of autophagy in the pathogenesis of COPD is important for the anti-COPD drug development.

Keywords COPD · Smoking · Emphysema · Bronchitis

39.1 Chronic Obstructive Pulmonary Disease

COPD is characterized by combination of persistent airflow limitation, small airway fibrosis, endobronchial goblet hyperplasia, and emphysema. The incidence of COPD is increasing year by year, and by 2020, COPD is expected to be the third most deadly disease in the world. The early stage of COPD is mainly characterized by chronic bronchitis. At this time, the pathophysiological changes are limited to small airways. The dynamic lung compliance reflecting lung tissue elastic resistance and small airway resistance declines. As the disease progresses, it gradually affects the airway, leading to pulmonary ventilatory dysfunction. The death of lung epithelial cells causes the damage of capillaries around the alveoli and results in a large decrease in blood flow among the alveoli. There are also some regions of the lung with normal blood perfusion, but the alveolar ventilation is poor and cannot support the gas exchange. Ventilation dysfunction can cause hypoxia and carbon dioxide retention,

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hypoxemia and hypercapnia, and eventually respiratory failure. The investigation of the pathogenesis of COPD proves that smoking is the primary pathogenic factor of COPD, especially in elderly male who smoke for a long time. In addition, the deletion of α1-antitrypsin is also an important cause of the onset of COPD. There are currently no clinical used drugs to reverse the lung function in COPD patients, but drugs and other treatments can significantly improve the life quality of patients. A study found the enhanced expression of autophagy markers in lung tissue of COPD patients, suggesting the role of autophagy in the pathogenesis of COPD. Thus, the studies which focus on the relationship between autophagy and the pathogenesis of COPD, especially smoking, α1-Antitrypsin, muscle atrophy, and autophagy are important (Alexandra et al. 2018).

39.2 Smoking and Autophagy

The study found that the number of autophagosomes in lung tissue of patients with COPD is increased, as well as the expression of LC3B-II, Atg4, Atg5-Atg12 complex, and Atg7. The chronic smoking model is the most commonly used COPD animal model in preclinical studies. The autophagy of lung is increased in C57BL/6 mice after 12 weeks of smoke exposure. The lung tissue sections showed an increase in the number of autophagosomes, and the expressions of multiple autophagy-related proteins are also enhanced. Knockdown of genes LC3B or beclin 1 had an inhibitory effect on smoke-induced COPD in mice, while autophagy signal deletion can inhibit cell death caused by Cigarette Smoke Extract (CSE). These evidences suggest that the autophagy signal is activated during the smoking process and the number of autophagosomes is increased. However, there are still different finding on the autophagy activity changes caused by smoking. After stimulation of different cells with bafilomycin A1 and CSE, the transformation of LC3B-I and LC3B-II illustrated that autophagic flux may be activated or inhibited by CSE in different cells (Chen et al. 2010).

Early studies generally suggested that oxidative stress and Reactive Oxygen Species (ROS) are the main causes of smoking-induced autophagy. ROS, as a signaling molecule that induces autophagy, activates a variety of autophagy pathways. Cigarette Smoke (CS) can promote the pro-oxidative state of epithelial cells, which in turn leads to oxidative stress and induces autophagy. CS-induced autophagy can be inhibited using the antioxidant *N*-acetylcysteine or NADPH oxidase inhibitor. Starvation is the most common method to activate autophagy with increased intracellular ROS levels during starvation, and this change can be inhibited by antioxidants. Thus, oxidative stress is not a specific agonist of CS-induced autophagy, but a universal autophagy-inducing biological mechanism. Furthermore, ROS-induced cell death can also be inhibited by knocking down autophagy-related genes (beclin 1, Atg4, Atg5, or Atg7), suggesting that ROS may mediate cell death by over-activation of autophagy (Bi et al. 2019).

The response protein early growth response-1(Egr-1) is an important factor regulating the transcription of LC3B. The expression of Egr-1 is up-regulated under stress, and the highly expressed Egr-1 is involved in cell apoptosis and inflammatory cascade. When CSE stimulates epithelial cells, Egr-1 rapidly binds to the LC3B promoter and induces LC3B transcription. Knockdown of Egr-1 can inhibit the autophagy induced by CS exposure. Interestingly, Egr-$1^{-/-}$ mice are resistant to autophagy activation and apoptosis induced by CS exposure and not sensitive to emphysema caused by CS exposure. Moreover, knocking out Egr-1 causes a physiological increase in the alveolar space of mice compared to wild-type mice. However, these effects may be caused by other biological effects regulated by Egr-1, including regulation of inflammatory cell infiltration, apoptosis signaling pathways (Morse and Rosas 2014).

The activity of autophagy may be related and resulting in dysregulation of ubiquitin-proteasome system activity during the pathogenesis of COPD, and numerous ubiquitinated proteins aggregate in the lungs of COPD patients mediated by CS exposure. Under normal conditions, intracellular ubiquitinated protein aggregates are often cleared by the autophagy pathway, but the number of intracellular ubiquitinated protein aggregates is increased at the onset of COPD, which suggests that impaired autophagy activity may be involved in the pathological changes of COPD. CS can promote the accumulation of p62 in cells, thus CS will cause damaged autophagic flux in the cells, and the accumulation of p62 can also be detected in lung tissue of COPD patients. The use of autophagy inducer carbamazepine not only reduces the number of intracellular protein aggregates induced by CS, but also inhibits CS-induced inflammatory responses and apoptosis.

In summary, autophagy is involved in the whole process of CSE-induced COPD pathogenesis, but the biological effects mediated by autophagy differ in different cells or at different stages of the disease (Haspel and Choi 2011). Using different models to study autophagy and the pathogenesis of COPD may lead to different conclusions. It is known that excessive activation of autophagy signals and inhibition of autophagic flux are important links in the pathogenesis of COPD. Over-activation of autophagy induced by ROS in epithelial cells causes cell apoptosis, and blocking autophagic flux may result in many misfolded proteins or ubiquitinated protein aggregates in cells. Autophagy activation and inhibition may occur in different regions in the same lung with COPD, so only normal autophagy function will not cause pathological changes (Fig. 39.1). CS contains more than 4700 components, including carbon monoxide, heavy metals, acetaldehyde, aromatic hydrocarbons, oxygen-free radicals, etc., which together regulate the onset of COPD. This caused great trouble for the study of CS-induced COPD. Current experimental methods are difficult to determine exactly which components of CS regulate autophagy activity. In addition, epidemiological data show that non-smokers can also have the same symptoms and pathological changes as CS-induced emphysema or COPD, suggesting that CS is not an absolute factor in the induction of COPD.

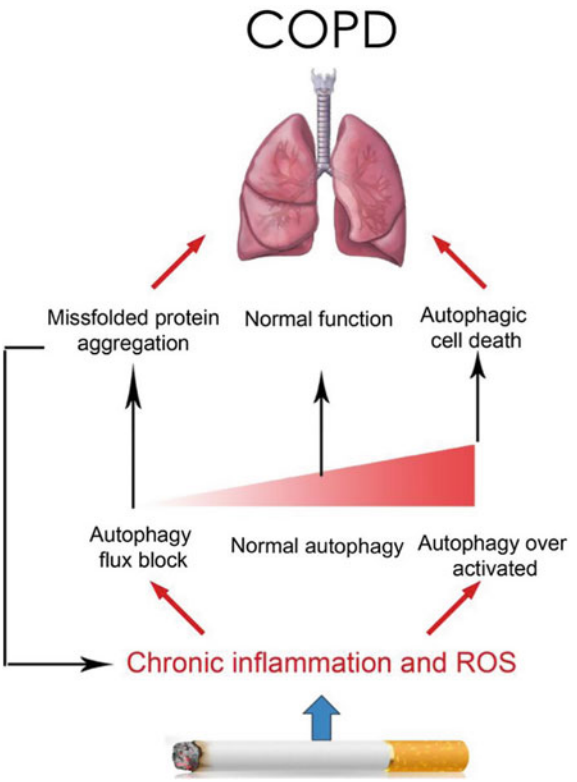

Fig. 39.1 Smoking regulates autophagy and promotes COPD. Long-term smoking can cause chronic inflammation and oxidative stress damage in the lung tissues. Oxidative stress and other factors cause excessive activation of autophagic flux in lung epithelial cells, that will induce autophagic death of lung cells, leading to emphysema. When the autophagy flux is blocked by chronic inflammation, the misfolded proteins and damaged organelles in the epithelial cells are accumulated, that accelerating the development of chronic inflammation

39.3 Autophagy Participates in Airway Remodeling in COPD

Airway remodeling is one of the characteristics of COPD, but the biological mechanism of its occurrence has not been fully determined. The pathological manifestations of airway remodeling are squamous changes in the pseudostratified ciliated columnar epithelium, cilia reduction, loss, and motor dysfunction, at the same time, the goblet cells in the airway enhancement, smooth muscle and fibrous connective tissue hyperplasia, and infiltration of a large number of inflammatory cells. Eventually, bronchial fibrosis may appear and emphysema occurs in the abnormal distal end of the bronchus. Smoking is the most important factor leading to COPD, which can produce a large amount of reactive oxygen species leading to the conversion of type I LCB to type II and the formation of autophagosomes in epithelial cells. Reactive oxygen species activates LC3B by increasing phosphorylation of JNK, and inhibition of reactive oxygen species can inhibit autophagy induced by smoking in airway epithelial cells. In another trending factor in COPD, neutrophil elastase can damage the bronchial epithelium and promote the progression of COPD. Neutrophil elastase

increases the level of PGF by Egr-1, thereby increasing the level of autophagosomes in damaged cells. Therefore, PGF in the serum of COPD patients can be used as a new drug target for autophagy treatment and drug discovery. In addition to smoking, environmental pollution is also an important cause of COPD. PM2.5 is a particle that can enter the respiratory tract with airflow and enter the circulatory system through the respiratory tract. Exposure of bronchial epithelial cells to PM2.5 increased the expression of LC3B, Beclin1, and VEGFA in cells, as well as the ratio of LC3BII/LC3BI and the number of autophagosomes (Zhu et al. 2018). 3-MA can inhibit the increase of VEGFA level caused by PM2.5, which proves that autophagy plays an important role in the VEGFA level enhancement caused by PM2.5. It is also involved in chronic inflammation and vascular remodeling caused by VEGFA. IL-13 is an important Th2 type cytokine involved in airway remodeling in the pathogenesis of COPD. IL-13-stimulated bronchial epithelial cells secrete elevated levels of MUC5AC and LC3B, knocking down Atg5 reduces secretion of MUC5AC in epithelial cells caused by IL-13, suggesting that IL-13 can induce autophagy to regulate airway epithelial cells secrete function.

39.4 Autophagy Regulates Apoptosis of COPD

Apoptosis of epithelial cells is an important mechanism of the pathogenesis of COPD, and apoptosis occurs in vascular endothelial cells, stromal cells, and inflammatory cells. The expression of apoptosis-related proteins caspase-3, Bax and Bad in lung tissue of COPD patients are increased, while the expression of anti-apoptotic protein Bcl-2 was unchanged, and these apoptosis-related proteins did not increase in the lungs of non-COPD smokers. There is evidence that even if smoking COPD patients quit smoking, their lung cells will still undergo apoptosis, so oxidative stress caused by smoking is not necessarily for lung cell apoptosis in COPD patients. Other causes that have been shown to induce apoptosis in COPD include protease/antiprotease imbalance and excessive inflammatory response.

Evidence suggests that activation of caspase-3 is only present in the lungs of patients with severe COPD (Global initiative for chronic Obstructive Lung Disease [GOLD] 4) but not in mild patient tissues (GOLD 0-2). However, all patients with COPD grade (GOLD 0-4) had elevated levels of autophagy markers in the lung tissue, including the expression of LC3B-II and the number of autophagosomes. This indicates that the change in autophagy activity during chronic exposure to CS is earlier than apoptosis. LC3B is the most classical marker protein of autophagy involved in CSE-induced epithelial cell apoptosis (Li et al. 2017). The number of apoptotic cells in the lungs of $LC3B^{-/-}$ mice was significantly lower than wild-type mice after CS exposure. CSE can induce apoptosis by inducing Fas-dependent Death-Inducing Signaling Complex (DISC) and activation of caspase-8. LC3B can form a complex with Fas and other death receptors in epithelial cells. LC3B is able to inhibit CSE-induced DISC formation. The LC3B-Fas complex formation is dependent on caveolin-1. In normal cells, caveolin-1 binds LC3B to form a trimer with Fas, thereby

inhibiting the formation of DISC by Fas. At the same time, LC3B can promote the dissociation of caveolin-1 and Fas. Knockdown of LC3B can enhance the interaction between caveolin-1 and Fas and further inhibit DISC formation to maintain cell survival.

39.5 Autophagy Regulates Macrophage Function to Participate in the Pathogenesis of COPD

Macrophages isolated from the lungs of COPD patients have generally lost their ability to phagocytose pathogens, but still are capable of releasing large amounts of pro-inflammatory cytokines. These phenomena ultimately lead to susceptibility to exogenous bacteria and bronchial inflammation in COPD patients. Analysis of autophagic flux in alveolar macrophages from patients with COPD over a 10-year history of smoking showed that autophagy was blocked after smoking. The specific analysis results show that the blockage of autophagic flux in macrophages is due to the slow degradation of autophagosome. In addition, Mitochondrial damage in macrophages may be an important cause of immune function loss.

Autophagy blockade in patients with COPD is also one of the reasons for the release of pro-inflammatory cytokines. Alveolar macrophages in COPD patients can release large amounts of IL-1β, causing terminal bronchial inflammation. This biological effect is composed of the following two factors. (1) Autophagy dysfunction in macrophages leads to activation of caspase-1 dependent on the Toll receptor linker protein TRIF, thereby promoting the production of IL-1β. Activated caspase-1 is able to regulate the release of IL-1β by its cleavage effect. Macrophages of Atg16L1-deficient mice produce large amounts of IL-1β and IL-18 compared to wild-type mice. Atg16L1/TRIF double knockout mice lost the ability to produce IL-1β form macrophage due to the inability to produce caspase-1. (2) The IL-1β gene first forms proIL-1β after transcriptional translation, and then pro-IL-1β hydrolyzes a short peptide by a specific serine protease to form the active cytokine IL-1β. In many cases, exogenous stimuli promote synthesis of pro-IL-1β in macrophages. Pro-IL-1β is activated and released when macrophages receive a second signal stimulus. Macrophages store pro-IL-1β in lysosomes before their activation. When autophagy is activated, pro-IL-1β is degraded in lysosomes, which in turn negatively regulates the release of IL-1β. The use of 3-MA or wortmannin to inhibit autophagy activity of macrophages can promote the activation of inflammation and the release of pro-IL-1β.

39.6 α1-Antitrypsin and Autophagy

α1-Antitrypsin (AAT) level dysregulation is an important cause of COPD in non-smokers. Autophagy can mediate clearance of aggregated AAT intracellularly. The "PiZ mutation" is the most common type of mutation in the AAT gene, which can cause misfolding during AAT translation and cause it to accumulate in the endoplasmic reticulum. The PiZ-mutated AAT cannot be transported from the liver cells into the blood, resulting in a disorder of the protease/anti-protease balance in the local tissues of the lungs, which ultimately induces the formation of emphysema. The ratio of LC3B-II/LC3B-I in the lung tissue of patients with AAT mutations is increased, indicating the changed level of autophagy. And the accumulation of PiZ-AAT mutant protein in hepatocytes is co-localized with LC3B. In ex vivo experiment, autophagy inhibitors can inhibit the degradation of exogenous AAT proteins. The use of autophagy agonists can reverse the spontaneous liver pathological changes in PiZ-AAT mutant transgenic mice, indicating impaired autophagy activity in local parts under the condition of AAT mutation. In addition, PIZ-AAT mutant protein aggregates were also detected in alveolar lavage fluid and tissue samples from COPD patients. The accumulation of PiZ-AAT mutant protein in alveolar macrophages suggests autophagy dysfunction in macrophages during the pathogenesis of COPD.

39.7 COPD Muscle Atrophy and Autophagy

In addition to lung damage, patients with COPD usually have symptoms such as weight loss, malnutrition, limited exercise capacity, and skeletal muscle atrophy. Skeletal muscle atrophy and muscle dysfunction are important factors affecting the life quality of COPD patients. The overall performance decreased muscle tone and endurance. The decrease of muscle mass in patients is mainly due to the imbalance of protein synthesis and degradation. A large number of early studies have shown that muscle atrophy in COPD patients is mainly caused by abnormal activation of the ubiquitin-proteasome pathway, until the role of autophagy lysosomal pathway in COPD skeletal muscle atrophy is gradually revealed (Guo et al. 2013). Autophagy is a very conservative energy-regulating mechanism of eukaryotic cells. There is evidence that basal autophagy activity is critical for the stability of skeletal muscle cells, which can be responsible for removing accumulated proteins and mitochondria in cells, as well as inducing muscle production in lower limbs after denervation.

The response of skeletal muscle to autophagy is different from other tissues in the stress response. After starvation for 24 h, autophagy responses in most tissue cells were activated, and the autophagy activity gradually decreased after 48 h of starvation. Skeletal muscle cells can still produce a large number of autophagosomes after 48 h of starvation. Knocking out Atg7 in skeletal muscle cells can completely inhibit the production of autophagosomes, leading to the accumulation of abnormal mitochondria and polyubiquitinated proteins, oxidative stress, and dysplasia. These

biological reactions can eventually cause muscle fiber degeneration. Similarly, Atg7 knockout mice can exhibit myasthenia gravis and significant muscle wasting symptoms. Inhibition of autophagy in skeletal muscle cells accelerates muscle atrophy caused by fasting and denervation. Similar to Atg7 knockout mice, Atg5-deficient mice also showed significant muscle atrophy. Studies have shown that BCL2-related Nutrient-Deprivation Autophagy Factor-1 (Naf-1) is an important regulator of skeletal muscle cell homeostasis. Mice lacking Naf-1 also showed muscle weakness and decreased muscle tone. Naf-1 null mice developed progressive gradual decline at 2–3 months of age, and sudden death at 12 months of age. Histone Deacetylases (HDACs) in skeletal muscle cells are also involved in autophagy regulated by starvation. Skeletal muscle-specific loss of HDAC1 and HDAC2 can lead to perinatal lethality in some animals. HDAC1/2 knockout mice cause autophagic dysfunction after birth and develop muscle lesions and muscle production/degradation disorders.

Nowadays, the correlation between skeletal muscle physical movement and autophagy has been gradually elucidated. There is evidence that proper physical movement can induce skeletal muscle autophagy. Moreover, physical movement can inhibit the interaction of BCL2 with Beclin-1, thereby activating autophagy signals. BCL2 mutant (BCL2 AAA) mice were unable to induce skeletal muscle autophagy through exercise. BCL2 AAA mice showed significantly declined tolerance to exercise, glucose metabolism disorder after exercise, and loss of protection for poor glucose tolerance induced by high-fat diet. Tolerant protection. It can be seen that autophagy plays an important role in regulating the metabolic response induced by exercise.

The vast majority of patients with COPD have symptoms of decreased skeletal muscle mass, which was initially suspected to be due to impaired overall autophagy. However, the autophagy activity of lung tissue and skeletal muscle in patients with COPD does not increase or decrease at the same time. It is worth noting that the levels of LC3B-II, Beclin-1, and p62 in skeletal muscle samples of COPD patients were significantly increased compared with healthy people. However, these phenomena do not indicate the true activation of autophagy, but may also be the result of autophagic signal activation and autophagic flux blockade. At present, many studies suggest that smoking is an important factor in the increase of autophagy in patients with COPD. However, it is unclear whether the change in autophagy activity induced by smoking is systemic or lung tissue specificity, and which components in CS regulate autophagy activity of skeletal muscle remains unclear. It is worth noting that the pathological changes in lung tissue of patients with COPD are mainly caused by CS, but the changes in autophagy activity of skeletal muscle in patients are mostly caused by malnutrition, which may be another theory independent of autophagy. Autophagy is a complex biological reaction process that intersects with various biological effects, including apoptosis, proliferation, inflammation, etc. The biological effects of autophagy in different tissues of the same individual are also different. Therefore, it is currently difficult to correctly define the role of autophagy in skeletal muscle atrophy in COPD patients.

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Chapter 40
Autophagy and Pulmonary Fibrosis

Xiaoxi Lv, Ke Li, and Zhuowei Hu

Abstract Pulmonary fibrosis is a progressive chronic inflammatory disease with a poor clinical outcome. Although pirfenidone and nintedanib have been approved by FDA to treat idiopathic pulmonary fibrosis (IPF), these drugs can only slow the progression of IPF. Autophagy plays an important role in the pathogenesis of pulmonary fibrosis. Whether the autophagic flux is blocked or not is directly related to the development direction of pulmonary fibrosis. Defining how autophagy activity regulates the pathogenesis of pulmonary fibrosis will greatly advance the progression of pulmonary fibrosis therapy.

Keywords Idiopathic pulmonary fibrosis · Autophagic flux · Oxidative stress · Collagen · Fibroblast

40.1 Pulmonary Fibrosis is a Chronic Inflammatory Disease

Idiopathic pulmonary fibrosis (IPF) is a type of chronic lung disease commonly found in the middle-aged and elderly population. The most important pathological change is the formation of scars in the lung parenchyma. When normal lung tissue is replaced by fibrotic tissue, the ventilatory function is significantly reduced, and lung compliance is also reduced. At present, the prevalence rate of pulmonary fibrosis in China has increased year by year, and the 5-year survival rate after diagnosis is about 30%, and there is no effective therapy in clinic. The causes of pulmonary fibrosis include smoking, environmental pollution, environmental dust, viral infections, bacterial infections, acid reflux, adverse drug reactions, and so on. These stimulating factors can cause alveolar epithelial cell damage, which in turn causes chronic

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inflammation. Tissue reconstruction is an important part of wound repair, but excessive reconstruction or imbalance of extracellular matrix formation and degradation can lead to tissue fibrosis. After acute and chronic lung injury, epithelial–mesenchymal transition (EMT) occurs in lung epithelial cells, and the damage itself may again cause oxidative stress damage to the lung tissue. Besides, large amounts of fibroblasts are activated in situ, while inflammatory cells infiltrate into the lesion and release a large number of inflammatory factors. Cross—or parallel interaction of multiple factors results in excessive deposition of extracellular matrix and pulmonary fibrosis. In 2011, we first reported that the immune factor IL-17A promotes the development and progression of pulmonary fibrosis by inhibiting the autophagy activity of lung epithelial cells; blocking IL-17A signaling inhibits pulmonary fibrosis by activating autophagy, which accelerates the clearance of lung tissue inflammation, and promotes the degradation of extracellular matrix (Mi et al. 2011). Interestingly, we have recently found that the pattern recognition receptor TLR2 or TLR4 differentially regulates autoimmune activity and autophagy activity, thereby promotes or inhibits the development of pulmonary fibrosis (Yang et al. 2012) (Fig. 40.1).

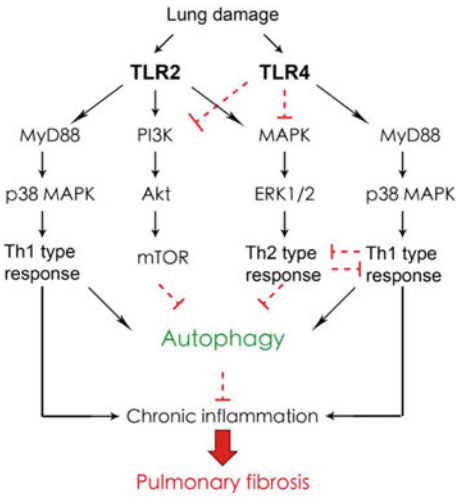

Fig. 40.1 TLR2 and TLR4 can be activated by different acute or chronic injuries, and induce biological effects through MyD88-dependent and -independent pathways. TLR2/TLR4 activates the MyD88-dependent pathway to directly stimulate the Th1-type immune response, causing lung damage. In addition, TLR2 activates the PI3K/Akt signaling pathway and induces a Th2-type immune response to inhibit autophagy activity, resulting in the inability to clear damaged organelles, misfolded proteins, and fragments in cells. If the inflammation mediated by TLR2 cannot be inhibited in time, persistent chronic inflammation and ineffective autophagy will promote the continuous development of pulmonary fibrosis. Unlike TLR2, TLR4 could both inhibit the PI3K/Akt signaling pathway and MAPK/ERK1/2-mediated Th2-type immune response, and induce a Th1-type immune response. These characteristics of TLR4 can activate autophagy at multiple levels, clear chronic inflammation, and promote the resolution of pulmonary fibrosis

These studies not only suggest that autophagy activity mediates the development and progression of pulmonary fibrosis by regulating immune-inflammatory response, but also suggest that regulation of lung tissue immune balance can correct the autophagy of dysregulated cells and thus achieve the purpose of preventing and treating pulmonary fibrosis. With the continuous exploration of the biological mechanism of autophagy, people gradually realize that autophagy can regulate the pathogenesis of pulmonary fibrosis at various levels, and most of the factors that promote pulmonary fibrosis, such as oxidative stress, endoplasmic reticulum stress, and hypoxia, can induce autophagy, but some viral infections can both inhibit autophagy and induce pulmonary fibrosis. More importantly, studies have shown that regulating autophagy activity may also be a new target for the treatment of pulmonary fibrosis (Haspel and Choi 2011).

40.2 Pulmonary Fibrosis and Autophagic Flux

Different from the regulation of autophagy activity in the lungs of patients with COPD, the lung tissue of patients with IPF shows obstructed autophagic flux and decreased autophagy function which is characterized by the decrease in the number of autophagosomes in the pulmonary fibrosis tissue, and the inability of the autophagosome to fuse with the lysosome, and the accumulation of p62 and ubiquitinated proteins in the cells. These phenomena are a pathological change that the organism gradually forms under the trend of long-term chronic inflammation. When the lung tissue has just developed an inflammatory response rather than a fibrotic lesion, the autophagy is activated, and a large number of autophagosomes and autophagosomes form to clear the intracellular pathogenic substances. When the lung tissue appears fibrotic lesions, the autophagic flow is blocked, the autophagy function is inhibited, and the formed autophagosomes and autophagic lysosomes cannot be removed. After the autophagy is inhibited, the main feature in lung tissues is that the pathogenic protein and the extracellular matrix cannot be removed, thereby the tissue fibrosis cannot be returned. This phenomenon also indirectly indicates that autophagy activity plays a protective role in the body against acute inflammatory reactions, but when chronic inflammation occurs, autophagic flux is inhibited for various reasons, and autophagy function impairs the body's inflammatory response, which promotes organizational restructuring (Anudeep et al. 2018) (Fig. 40.2).

Autophagy, in addition to regulating cellular metabolic balance, has also been shown to prevent cell senescence. Decreased autophagy in alveolar epithelial cells promotes p21-mediated cellular aging. Aging lung epithelial cells can release a large number of profibrotic cytokines and reversely inhibit autophagy, which promotes the development and progression of pulmonary fibrosis. At the same time, a large amount of ubiquitinated protein accumulated in the epithelial cells cannot be removed, which induce ROS and endoplasmic reticulum stress, and exacerbate the inflammatory response in the lungs. We have recently discovered that the anti-allergic drug rupatadine exhibits the anti-pulmonary fibrosis effect by inhibiting PAF-induced

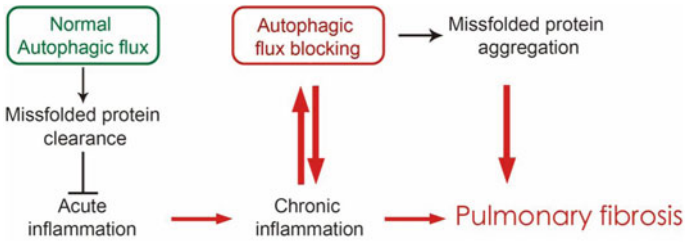

Fig. 40.2 Blocking autophagic flux promotes pulmonary fibrosis. Autophagy activity is a key factor in maintaining the immune stability of epithelial cells. Normal autophagy can promote the clearance of pathogenic proteins and damaged organelles, and inhibit acute inflammatory responses. However, when pulmonary fibrosis occurs, acute inflammation gradually turns into chronic inflammation due to the pro-inflammatory cytokines and inflammatory cells in damaged tissues. This sustained inflammatory response results in obstruction of epithelial autophagic flux and ultimately promotes pulmonary fibrosis by inducing accumulation of a large number of pathogenic proteins

lung epithelial cell senescence, activating autophagic flux in lung cells, and reducing the release of pro-fibrotic cytokines (Lv et al. 2013). Unlike epithelial cells, lung fibroblasts do not induce cell senescence after autophagy dysfuncton, and fibroblasts with autophagic flux blocked are more prone to their own activation, which may be related to cell specificity.

As an inhibitor of mTOR, and rapamycin is used to inhibit bleomycin-induced pulmonary fibrosis in mice. In addition to regulating autophagy, mTOR is involved in the regulation of body protein and fat anabolism, cell growth, and other aspects. Inhibition of these biological reactions by rapamycin can also affect the differentiation of fibroblasts and the production of extracellular matrix, thus not precluding rapamycin in the treatment of pulmonary fibrosis in the presence of autophagy-independent pathways. Moreover, rapamycin has entered the phase I clinical trial stage for the treatment of pulmonary fibrosis, and more anti-pulmonary fibrosis drugs targeting autophagy activity are in the research and development stage.

40.3 Oxidative Stress Regulates Autophagy and Participates in the Pathogenesis of Pulmonary Fibrosis

The endoplasmic reticulum is an important organelle that mediates the folding and transport of newly synthesized proteins. Endoplasmic reticulum stress-induced autophagy has significant cell specificity. Activation of autophagy by endoplasmic reticulum stress in colon and prostate cancer cells can reduce cell death, but promotes cell death in normal human colon cells and embryonic fibroblasts. Inducing autophagy through endoplasmic reticulum stress in lung fibroblasts can induce non-folded protein response, thereby compensating for biological dysfunction caused by

endoplasmic reticulum stress. Fibroblasts degrade misfolded proteins by autophagy lysosomal pathway, indicating that autophagy is an important protective mechanism for lung fibroblasts. Mutations in surfactant protein C in patients with familial hereditary pulmonary fibrosis can lead to misfolded protein accumulation, endoplasmic reticulum stress, and apoptosis of type II alveolar epithelial cells (AEC II). Non-genetic IPF patients with no mutations in SFTRC show a significant endoplasmic reticulum stress in lung tissue, but endoplasmic reticulum stress in lungs of IPF patients did not activate autophagy. The large amount of autophagosomes accumulates in fibroblasts and epithelial cells and the autophagic flux of lung cells is blocked. So, autophagy cannot compensate for the accumulation of misfolded proteins caused by endoplasmic reticulum stress, and finally cause AEC II death.

Similar to endoplasmic reticulum stress, oxidative stress can also regulate autophagy activity (Mizumura et al. 2012). There is evidence that moderate oxidative stress-induced autophagy activation can promote clearance of damaged organelles and maintain cell survival. The dysregulation of autophagy induced by acute and sustained oxidative stress can lead to increased intracellular ROS, lysosomal membrane damage, and apoptosis. The levels of ROS and oxidative stress markers are increased in lung tissue of patients with IPF, and the level of ROS is negatively correlated with lung function. Excessive production of ROS can provide a favorable tissue microenvironment for fibrotic pathology while causing lung damage. Acetylcysteine (NAC) is a first-line drug for clinical treatment of pulmonary fibrosis, which promotes the synthesis of glutathione and acts as an antioxidant. It has also been shown to reverse regulate ROS-induced autophagy.

Hypoxic-inducible factor 1-α (HIF1-α), which is also expressed during the pathogenesis of IPF, can also regulate autophagy activity. The HIF1-α target gene BNIP3 is involved in hypoxia-induced autophagy, and its expression is increased in lung tissue of IPF patients with chronically hypoxic. BNIP3 can break the Bcl2/beclin 1 interaction, promote the formation of autophagy core complexes, and ultimately lead to cell death by inducing autophagy activation. It has been observed that hypoxia-induced apoptosis of alveolar epithelial cells occurs only in the early stages of pulmonary fibrosis, or in the lungs of normal and lesion margins in patients with pulmonary fibrosis, rather than in established fibrotic tissue. This indicates that autophagy induced by hypoxia may participate in and regulate the initial stage of fibrosis. When tissue fibrosis is formed, autophagy activity is inhibited, and cells no longer undergo apoptosis.

40.4 Viral Infection Participates in the Pathogenesis of Lung Fibrosis by Inhibiting Autophagy

Viral infection is another cause of pulmonary fibrosis. Herpes virus, adenovirus, hepatitis C virus, Epstein–Barr virus, etc. can gradually induce pulmonary fibrosis while causing chronic infection of the body. Autophagy is an important mechanism

that mediates the body's fight against viral infection and escape. Cells infected with virus can enhance the level of autophagy by upregulating eIF2a phosphorylation. Autophagy lysosomes and phagosomes together constitute a defense system for host cells to eliminate viruses. However, most viruses can inhibit autophagy by inhibiting the formation of autophagy core complexes, ultimately resulting in immune escape. For example, Epstein–Barr virus can synthesize a large amount of Bcl-2 analog in type II alveolar epithelial cells. Unlike the wild-type Bcl-2 protein, these Bcl-2 analogs are not themselves phosphorylated by JNK due to the specificity of their protein secondary structure. The Bcl-2 analog that loses its phosphorylation ability can form a stable complex with the autophagy core complex key protein beclin 1 to produce a sustained autophagy inhibitory effect. Thus, inhibition of autophagy activity is not only the result of chronic inflammation, but also the cause of immune escape of pathogenic viruses. Thus, inhibition of autophagy activity promotes the development of pulmonary fibrosis by various mechanisms at multiple levels.

40.5 Autophagy and Collagen

The most important pathological change of pulmonary fibrosis is the massive accumulation of extracellular matrix. Activated myofibroblasts secrete a large amount of type I and type III collagen fibrins in the peribronchial, alveolar wall, and interstitial lung. These two kinds of collagen are important components of the connective tissue of the body, but the imbalance of the formation and degradation of extracellular matrix during chronic inflammation leads to the occurrence of tissue fibrosis. The deposition of type I and type III collagens in the pathogenesis of pulmonary fibrosis is related to the degree of onset. Type III collagen mainly accumulates in less serious patient, while type I collagen accumulates in patients with severe pulmonary fibrosis.

In order to induce pulmonary fibrosis recovery, it is necessary to remove collagen which is excessively deposited in the lungs. In this process, multi-polymerized collagen is first decomposed into collagen monomers or small fragments by a protease degradation system such as extracellular matrix metalloproteinases (MMPs), and then macrophages and fibroblasts can phagocytose these collagen molecules or fragments which causing their degradation within the cell. Macroautophagy is an important biological process in which cells nonselectively degrade long-lived proteins, organelles, and regulate cell nutrition and energy. During the recovery process of pulmonary fibrosis, autophagy plays an important role in the degradation of pathogenic proteins, misfolded proteins, and extracellular matrices (Kiichi et al. 2016). If the autophagy remains active, the damaged area can return to the correct direction. However, when autophagy is inhibited, it may lead to persistent inflammation and more serious pathological changes.

By using autophagy inhibitors and proteasome inhibitors, it has been found that intracellular collagen can be degraded by the autophagic lysosomal pathway. After activation of fibroblasts with adrenaline, degradation of intracellular collagen, and activation of autophagy can be observed simultaneously. Another study found that

autophagy was inhibited in primary cultured fibroblasts from IPF after co-culture with multimeric collagen, whereas autophagy was activated by co-culture of healthy human primary fibroblasts with multimeric collagen. In patients with pulmonary fibrosis, Akt/mTOR activates after exposure to collagen, resulting in inhibition of autophagy, making it impossible to remove intracellular clearance after phagocytosis of collagen fragments or monomers. And a large body of evidence indicates that fibroblasts with autophagy inhibition can release more collagen fibers and aggravate the development of pulmonary fibrosis (Fig. 40.3). The impaired autophagy function in $LC3^{-/-}$ mice significantly increased the collagen and the inflammation in the lungs after bleomycin injury and decreased the survival rate, whereas the activation of autophagy in mice with bleomycin injury can reduce collagen accumulation in the lungs and increase survival rate. Beclin 1 is an important component of the autophagy core complex, and mice lacking beclin 1 exhibit multi-organ fibroproliferative properties with extracellular matrix deposition (Ricci et al. 2013). Similarly, knocking down beclin 1 in fibroblasts also leads to an increase in type I collagen levels (Principe et al. 2012).

Bafilomycin A1 can inhibit autophagosome fusion with lysosomes to block autophagic flux. Inhibition of autophagic lysosomal pathway using bafilomycin A1 revealed an increase in type I collagen in fibroblasts and colocalization with the lysosomal markers LAMP-1 and LC3B, but did not alter the mRNA level of type I collagen. Activation of autophagy with trifluoperazine alleviated TGF-induced increase in collagen content in fibroblasts, but did not affect collagen transcription. These

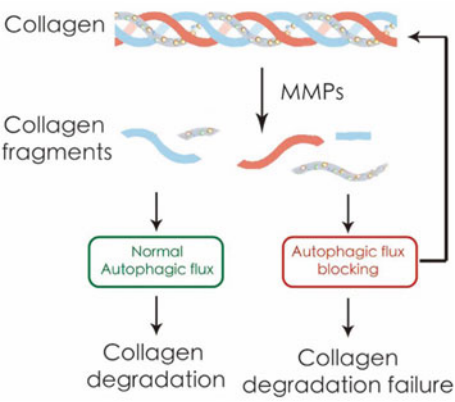

Fig. 40.3 Blocking autophagic flux in epithelial cells inhibits collagen degradation. Extracellular matrices, particularly polycolloidal collagen, are first degraded into collagen fragments by the action of matrix metalloproteinases. These collagen fragments are then phagocytosed by macrophages and fibroblasts, which are then degraded by the proteasome and autophagic lysosomal systems. During the pulmonary fibrosis, the autophagic flux in fibroblasts and macrophages is blocked, and the cells cannot degrade the collagen fragments after phagocytosis. At the same time, fibroblasts blocked can release more collagen molecules to the local damaged tissue, aggravating the onset of the disease

above evidences suggest that autophagy is an important pathway for the degradation of intracellular collagen, and inhibition of autophagy or blocking of autophagic flux leads to intracellular collagen clearance failure. In addition to tissue fibrosis, some other cell biology studies have provided theoretical support for the process of autophagy to degrade collagen. Hsp47 is a collagen-specific molecular chaperone. The deletion of Hsp47 can lead to the cell type I procollagen not being correctly folded or misfolded to form aggregates. These aggregates were finally confirmed to be degraded by the autophagolysosomal pathway. As a classic tissue-promoting factor, TGF-β can not only induce the production of fibronectin and collagen, but also inhibit the degradation of extracellular matrix by MMPs. However, autophagy is activated after TGF-β receptor activation. Some scholars believe that TGF-β promotes fibroblast synthesis of collagen and promotes intracellular degradation of collagen molecules by activated autophagy, but some people believe that TGF-β only activates the autophagy signaling pathway but not activates autophagic flux.

40.6 Autophagy and Fibroblasts

The basic autophagy activity is an important biological mechanism for maintaining fibroblast survival, and the autophagy activity of fibroblasts in most tissues in the body can be maintained at a high level, such as lung fibroblasts, hepatic stellate cells, and cardiac Fibroblasts, dermal fibroblasts, and mesangial cells. Infiltration of fibroblasts and activation of myofibroblasts are important steps in the pathogenesis of pulmonary fibrosis. In the pathogenesis of pulmonary fibrosis, in addition to the fibroblasts collected by the circulatory system to the injured tissue, there are also tissue in situ fibroblasts that activate and release a large amount of collagen molecules to cause fibrotic pathological changes. Knockdown of LC3B or beclin 1 enhanced the activation of myofibroblasts induced by TGF-β and increased the expression of fibronectin and α-SMA in myofibroblasts. Blocking ATG5 by genetic means in fibroblast causes autophagic flux blockade which promoting their differentiation into myofibroblasts and releasing large amounts of extracellular matrix. In addition, the application of autophagy agonists in the mouse model of pulmonary fibrosis induced by bleomycin can alleviate the level of pulmonary fibrosis in mice. After prolonged starvation, the expression of LC3B-II is increased, the content of p62 is decreased in fibroblast, and the cells showed obvious activation characteristics of myofibroblasts. At the same time, the expression of α-SMA in the cells is increased, and the contents of type I and type III collagens were significantly increased, and a large number of stress fibers appeared in the cytoplasm. The use of autophagy inhibitors or knockdown of ATG7 can inhibit starvation-induced myofibroblast activation. Autophagy-activated fibroblasts after starvation induction can release a large amount of CTGF to promote collagen production, while knocking down CTGF can inhibit myofibroblast activation. In addition, at the initial stage of starvation, Akt phosphorylation level in fibroblasts is decreased, autophagy is activated. However, when fibroblasts are starved for more than two days, Akt exhibits spontaneous phosphorylation, at which

time the cells are at the level of autophagy activation, and their mTOR/Akt pathway It is still active. At this time, the use of rapamycin inhibits myofibroblast activation, suggesting that autophagy and the mTOR/Akt pathway play a relatively independent role in starvation-induced myofibroblast activation.

40.7 Autophagy and Epithelial Mesenchymal Transition

EMT is one of the important pathogenesis of pulmonary fibrosis. Alveolar epithelial cells lose epithelial cell characteristics under stress stimulation, which is characterized by the decrease of E-cardherin expression and the increase of vimentin expression, thus inducing cytoskeleton and morphology with mesenchymal features. Alveolar epithelial cells that develop EMT lose cell polarity, lose their attachment to the basement membrane, and transform into fibroblasts to repair tissue damage caused by trauma and inflammation. However, when the inflammatory response persists, the EMT process persists and eventually promotes the development and progression of pulmonary fibrosis. Tumor cells can induce autophagy after starvation treatment, and can also activate TGF-β/Smad3 signaling pathway to induce EMT response in cells. The use of the autophagy inhibitor chloroquine or knockdown of ATG3 or ATG7 can inhibit the EMT response induced by starvation (Singh et al. 2015). Tunicamycin, an endoplasmic reticulum stress inducer, can induce EMT response while increasing the expression of LC3B-II and beclin 1. Inhibition of autophagy by using 3-MA or bafilomycin A1 suppresses EMT caused by tunicamycin. In addition, many scholars have found that not only autophagy activation can induce EMT reaction, but also epithelial cells can induce EMT production when autophagic flux is blocked and autophagy is impaired in some cases. After the autophagic flux is blocked in epithelial cells, some nuclear factors that can induce EMT, such as Twist, cannot be cleared by autophagy, leading to accumulation of Twist in cells and causing EMT response. In addition, epithelial cells with autophagic flux blocked can cause chronic inflammation due to the inability to clear large amounts of misfolded proteins, which is one of the causes of EMT in epithelial cells. Endothelial cells are important physiological barriers in human lung tissue. In addition to protecting blood vessels, they also function as nutrient exchanges. Endothelial-to-mesenchymal transition (EndMT) is also an important biological mechanism in the pathogenesis of pulmonary fibrosis, and an important source of local myofibroblasts in injured tissues. In cells undergoing EndMT, the expression of α-SMA, N-cadherin, and collagen in endothelial cells increased significantly, while the expression of endothelial markers CD31 and VE-cadherin decreased. It has been proved that the inhibition of autophagy activity is involved in the process of endothelial mesenchymal transition. Knockdown of ATG7 can activate TGF-β signaling pathway and upregulate the expression of pro-fibrotic genes. Endothelial cell-specific ATG7$^{-/-}$ mice have significantly reduced endothelial cell characteristics and are more sensitive to bleomycin-induced pulmonary fibrosis and collagen deposition. Normal autophagic flow activity prevents the development of EndMT and the development of tissue fibrosis.

40.8 Silicosis and Autophagy

Silicosis is a chronic fibrotic lung disease whose pathological changes are mainly caused by inhalation of free silica. At present, there are very limited methods for clinical treatment of silicosis, other than lung washing, and lung transplantation, there is no effective treatment strategy. The pathogenesis of silicosis is similar to that of pulmonary fibrosis. It is characterized by alveolar epithelial cell damage, fibroblasts differentiate and accumulate into myofibroblasts, epithelial mesenchymal transition, and excessive deposition of extracellular matrix proteins in the lung. The formation of silicotic nodular lesions leads to the formation of scar tissue in the lungs which destroys basic lung function and ultimately leading to respiratory failure. Silicosis has the highest incidence among workers exposed to dust, and the latest data show that the prevalence of silicosis is 0.89%. Therefore, identifying new targets for the prevention and treatment of silicosis is critical. Since autophagy is a highly conserved cellular process that regulates the turnover of cytoplasmic proteins via a lysosomal-dependent pathway, autophagy can be linked to degraded substrates that are over-deposited in silicosis, including fibronectin and intracellular secretion of collagen. In the mouse model of silica-induced pulmonary fibrosis, autophagy activity was decreased, and miR-449a level was downregulated, which was used as a new inhibitor of silica-stimulated lung fibroblast differentiation and pulmonary fibrosis in mice (Han et al. 2016). miR-449a enhances autophagy activity in vitro and in vivo. As a key protein of autophagy, Bcl2 is a target of miR-449a. miR-449a inhibits pulmonary fibrosis by regulating the expression of Bcl2 and autophagy activity, so regulation of miR-449a level may be an effective method for the treatment of fibrotic diseases caused by silica.

Silica nanoparticles (SiO_2NPs) are cytotoxic to different types of cells including cardiomyocytes and lung epithelial cells in vitro. Studies have shown that the uptake of silica nanoparticles by lung epithelial cells and the cytotoxic effects of the particles are closely related to autophagy. Electron microscopy images showed that the internalized nanoparticles were encapsulated in double-coated autophagic vesicles. PCR analysis of autophagy-related genes also showed enhanced autophagy activity in the presence of 20-nm silica nanoparticles. Although lung epithelial cells have a higher level of autophagosomes, the activity of caspase-3 does not increase, and mitochondrial cytochromes are not released. Therefore, the nanosilica particles induce the expression of stress-related genes, thereby triggering autophagy and protecting the cells from programmed cell death. Therefore, autophagy is a protective mechanism for silica particle stimulation itself, but it also causes fibrotic disease when autophagy is inhibited by chronic inflammation caused by damage to the local immune microenvironment.

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Chapter 41
Asthma and Autophagy

Xiaoxi Lv, Ke Li, and Zhuowei Hu

Abstract Asthma is one of the most common diseases of the respiratory system, with typical pathogenesis and pathological changes. The current research shows that autophagy is mainly involved in the pathogenesis of asthma by regulating the body's innate and adaptive immune responses. At the same time, a large number of epidemiological studies have shown that multiple autophagy genes affect the risk of asthma at the level of genetic polymorphism. This chapter will explore the relationship between autophagy and asthma.

Keywords Immune response · Viral infection · Gene polymorphism · Inflammation

41.1 Innate Immune Response and Adaptive Immune Response

Inflammation caused by asthma. The adaptive immune response is a series of biological effects mediated by antigen-dependent T helper cells (CD4+). Th1 cells secrete cytokines such as IFN-α and IFN-β, and Th2 cells mainly secrete cytokines such as IL-4, IL-5, IL-10, and IL-13, which together regulate the immune balance of the body. When the Th1/Th2 immune balance breaks, the body will have a corresponding inflammatory response. The immune response mediated by Th2 cells is a crucial starting factor for the pathogenesis of asthma. High levels of Th2-type immune responses can be detected in both animal models and clinical patient samples, and Th1/Th2 immune imbalance disorders can eventually lead to airway inflammation and exacerbate asthma attacks.

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The innate immune response is a conserved immune response. It is the first barrier against pathogens. It can specifically recognize pathogen-associated molecular patterns (PAMPs) on the surface of pathogenic microorganisms and trigger immune responses. Unlike adaptive immune responses, innate immune responses are initiated more rapidly, with a large number of immune cells and nonimmune cells involved. TLR is a pattern recognition receptor that links innate immunity and adaptive immunity, and both TLR2 and TLR4 are included in the development of asthma.

So far, the role of autophagy in innate and adaptive immune responses has become clearer (Jyothula and Eissa 2013). Autophagy can deliver antigen to MHC II molecules via lysosomal catalysis, a feature of autophagy that has been used in animal models to enhance the immune effect of BCG. Autophagy is essential for the development and survival of lymphocytes. Both autophagy-deficient B-cell and T-cell differentiation and immunomodulatory capacity are affected.

The number of peripheral T cells in the mice with conditionally deficient ATG5 was significantly decreased, and the proliferation of T cells was reduced considerably after antigen stimulation. Autophagy activity in Th2 cells is relatively high, and these cells can tolerate more severe nutritional deficiencies. The tolerance to autoantigen also requires autophagy, and the autoimmune activity of mice with thymus knockout ATG5 is enhanced, and its autoantigen triggers a robust immune response due to the loss of autophagy monitoring. The regulation of autophagy by Th1 and Th2 cytokines is opposed. IFN-γ promotes autophagy activity, while IL-4 and IL-13 inhibit autophagy activity. Electron microscopic evidence shows an increase in the number of autophagosomes in bronchial tissue fibroblasts and epithelial cells in asthmatic patients (Haspel and Choi 2011). At the same time, researchers are gradually illustrating the relationship between the increase in autophagosomes and the autophagic flux.

41.2 Autophagy and Inhaled Viral Infection

Autophagy is an essential link between inhaled infection and asthma. Inhaled viral infection in adolescents is an important risk factor for asthma attacks. Epidemiological evidence suggests that viral infections in the lower respiratory tract in infants are closely related to the incidence of asthma. Inhaled viral infection is directly linked to acute exacerbations in most asthmatic populations (85% of adolescents, 80% of adult patients). Rhinovirus (HRV), syncytial virus (RSV), influenza virus, coronavirus, and adenovirus are often detected in the airway of patients with acute exacerbation of asthma. These viral infections can increase the inflammatory response in the lungs and increase the number of Th2 cells. The release of factors also increases the airway hyperresponsiveness of the patient. HRV2 can stimulate autophagy activation, and viral replication is increased when autophagy is activated, while viral replication is also inhibited after autophagy activity is blocked using autophagy inhibitors. RSV

infection of mouse dendritic cells induces autophagy activation and also promotes the release of IFN-β, TNF-α, and IL-6. The release of these cytokines is dependent on autophagy activity.

Autophagy also supports the interaction between antigen-presenting cells and T cells in the process of viral infection, thereby promoting the activation and maturation of dendritic cells. Asthmatic patients are also more susceptible to the influenza virus, and the number of autophagosomes in the lung tissue of asthma patients infected with H5N1 virus can be observed by electron microscopy. At the same time, in vitro experiments also proved that the H5N1 virus-infected lung epithelial cells autophagy activation and the emergence of autophagic cell death phenomenon. Inhibition of autophagy can attenuate H5N1-induced acute lung injury. After activating autophagy by coronavirus infection, increased nonstructural protein (ns6) in cells can produce a large number of autophagosomes through the endoplasmic reticulum effect.

The interaction of the adenoviral protein E1B19K with the beclin1/PI3KC3 complex results in increased PI3KC3 activity leading to autophagy. When the autophagy of the host cell is induced and activated, the pathogen can be encapsulated by the autophagosomes to promote its own reproduction in the cell. On the other hand, autophagy is also an important way for the body to protect from viruses. During pathogen infection, host cells can clear pathogens in cells by autophagy. This contradictory biological effect may be related to the specificity of the virus being infected, and also to the immune microenvironment in which the body itself is located. A variety of inhaled viral infections that induce asthma attacks can produce a Th2-type immune response, and the resulting Th2-type cytokines can mediate inhibition of autophagy activity. In fact, the inhibition of autophagy is partly blocked by the flow of autophagy, and this is one of the reasons why the virus escapes from the autophagy in the cell. In summary, autophagy mainly regulates the replication and proliferation of inhaled viruses in host cells during the pathogenesis of asthma. The immune suppression induced by the virus avoids its elimination by autophagy (Mabalirajan 2017).

41.3 Autophagy Gene Polymorphism and Asthma

A large number of studies including genome-based sequencing and genomics-based studies have identified susceptibility genes to increase the risk of asthma; many genes are involved in and regulate the pathogenesis of asthma, including some autophagy-related genes. A study which included 1338 patients with asthma explored the genetic role of autophagy-related genes such as ULK1, p62, LC3B, beclin1, and ATG5 in asthma attacks. Studies have shown that the mRNA expression of ATG5 in nasal epithelial cells is significantly increased in patients with acute asthma attacks. After genetic polymorphism studies, it was found that the rs12212740 gene locus of ATG5 is involved in the pathogenesis of asthma, which is located in intron 3 of the ATG5 gene. The allele *G* of rs12212740 was identified as a risk factor for asthma, and this allele was also positively correlated with the decline in FEV1.

Another study including about 600 clinical samples also demonstrated that the ATG5 gene polymorphism is closely related to childhood asthma attacks. The results showed that rs12201458 allele *A* in ATG5 could reduce the risk of asthma, while ATG5 rs510432 allele *G* can increase the risk of asthma. Unlike ATG5 rs12212740, rs510432 is located upstream of the first exon of the promoter. Through luciferase experiments, researchers found that STAT1 and C-FOS are essential transcription factors that activate the rs510432 *G* allele. Both transcription factors are increased in the pathogenesis of asthma and have been shown to promote the onset of asthma. The phagocytosis is likely an effector element during the asthma attack. However, how the autophagy reaction ultimately regulates the onset of asthma remains to be further studied.

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Chapter 42
Autophagy and Others Respiratory Diseases

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Abstract Besides COPD, pulmonary fibrosis, and asthma, autophagy also participates in the development of many other respiratory diseases. Cystic fibrosis is an innate lung disease. Unlike idiopathic pulmonary fibrosis, cystic fibrosis has unique pathogenesis. Autophagy is an essential biological mechanism for the removal of misfolded proteins and damaged organelles in cells. Abnormal autophagy activity is involved in the pathogenesis of cystic fibrosis. Various studies have demonstrated that abnormalities or impaired autophagy are associated with cardiovascular diseases including pulmonary vascular disease. Autophagy plays a key role in maintaining normal vascular biological functions and vascular cell tissue homeostasis, and also plays an important role in the pathogenesis of various vascular diseases. For example, recent studies have found that autophagy participates in the occurrence and development of pulmonary hypertension. In addition, autophagy plays a central role in both innate and adaptive immune responses in immune cells or other cells with immune function. Thus, autophagy is the important cellular biological mechanism which causes cell fighting against pathogenic microorganisms including viruses, bacteria, and parasites. In this chapter, we discuss the work related to autophagy and other lung diseases.

Keywords Cystic fibrosis · Pulmonary hypertension · Infection · Obstructive sleep apnea

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42.1 Autophagy and Cystic Fibrosis

42.1.1 Cystic Fibrosis is a Hereditary Lung Disease

Cystic pulmonary fibrosis (CF), also known as the viscous obstructive disease, is a congenital disease with a family autosomal recessive inherited disease. This disease is more common in the lungs but can also occur in the pancreas, liver, kidneys, and intestines; the main symptoms of the lungs are difficulty in breathing and coughing. CF has a high incidence in North America, with less frequency in Asia and Africa. According to pathological changes, CF belongs to the exocrine gland disease, and its primary cause is the CFTR gene mutation. CFTR mainly regulates the body to produce sweat, digestive fluid, and mucus. When the CFTR function is disordered, the airway surface fluid is decreased, the acid glycoprotein content in the mucus gland secretion is also increased. In addition, the secondary infection is the primary pathological basis of the patients with CF. Recurrent episodes of atelectasis and secondary infections eventually lead to extensive fibrosis and obstructive emphysema in the lungs.

42.1.2 Glutamine Transaminase 2 Regulates Autophagy in Cystic Fibrosis

The most common mutant site of CFTR is the 508 site (phenylalanine) deletion. This CFTR mutant has a tendency to aggregate and therefore tends to be prematurely degraded in protein processing in the endoplasmic reticulum. Autophagy is an important pathway for cells to clear misfolded proteins and damaged organelles. It has also been proved to be one of the important biological mechanisms of the pathogenesis of cystic fibrosis.

Glutamine transaminase 2 (TG2) is a direct cross-linking enzyme that catalyzes acyl transfer reactions in the cell and induces cross-linking within or between proteins by inducing a series of biochemical reactions. TG2 in airway epithelial cells which deficient CFTR occurs SUMO modification. SUMO modification is one of the fundamental post-transcriptional modification mechanisms of proteins, which can regulate the transcriptional activity, nuclear translocation, and biological functions of proteins. TG2 has three SUMOylated lysine sites, which can inhibit TG2 from ubiquitination and inhibit TG2 degradation after SUMOylation. Increased ROS content in CF epithelial cells leads to increased expression of the E3 ligase PIASy, which mediates TG2 SUMOylation, ultimately leading to an increase in intracellular TG2 protein levels. There is evidence that CFTR mutants can induce large amounts of ROS in the lung epithelial cells and trigger endoplasmic reticulum stress, and the autophagy pathway is activated (Fig. 42.1). Moreover, the interaction between beclin1 and Bcl-2 is weakened in CF epithelial cells, indicating that the microenvironment in CF cells is conducive to autophagy activation. However, high expression and

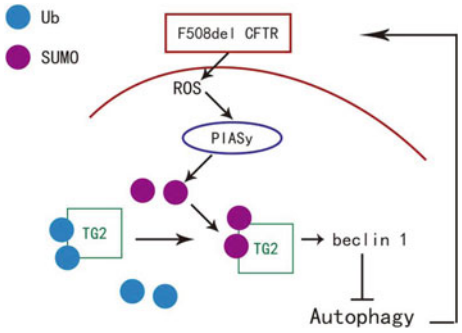

Fig. 42.1 The CFTR mutant inhibits autophagy in cells. The production of intracellular ROS is increased in CFTR mutated cells, and ROS can induce an increase in the expression of E3 ubiquitin ligase PISy. PISy inhibits TG2 degradation through the ubiquitin-proteasome pathway by causing SUMOylation of TG2. The large amount of TG2 accumulated in the cells crosstalk with beclin 1, which ultimately affects the formation of autophagy core complex and inhibits autophagy activity. After inhibition of autophagy, CFTR mutants cannot be degraded by autophagy pathway

sustained activation of TG2 can promote the crosstalk between beclin1, which leads to the decreasing stability of autophagy core complex and inhibition of autophagy activity. After autophagy was inhibited, the damaged mitochondria and p62 appeared to be accumulated. At the same time, the CFTR mutant could not be cleared by the autophagy pathway, which caused more ROS in the cells to induce a vicious circle. Overexpression of beclin1 in CF epithelial cells restores autophagy activity in cells. Similarly, intratracheal instillation of beclin1 lentivirus in CFTR mutant mice increases the number of LC3 puncta in cells and reduces the level of p62 in epithelial cells. It can be seen that the dysfunction of autophagic induces the onset of CF which depends on the formation of autophagy core complex.

At present, impaired autophagy in epithelial cells has become a new target for CF therapeutic research (Junkins et al. 2014). Cysteine stimulates CF epithelial cells to increase the maturation of CFTR mutants, promotes the mutant entry into the Golgi apparatus to exert their original biological activities, and increases the stability of CFTR on the cell membrane surface. After cysteine treatment, autophagy activity in CF epithelial cells is increased, which may be a result secondary to the recovery of CFTR function. After restoring CFTR function, the level of SUMOylation of TG2 and intracellular ROS is decreased, the level of cross-linking of beclin1 is reduced, and the stability of autophagy core complex is increased, thus leading to the recovery of autophagy activity. In the normal bronchial epithelial inhibition of CFTR function, the mature glycosylated CFTR on the cell membrane is reduced by 2/3, which also leads to an increase in intracellular ROS content, which induces a decrease in the stability of the TG2-mediated autophagy core complex, and this phenomenon disappears after the cysteine treatment (Luciani et al. 2010). It has been shown that cystine does not directly activate autophagy. If cystine-induced cell surface CFTR stability persists, the ROS/TG2 pathway in CF epithelial cells cannot inhibit autophagy. At the same time, cystine-treated CF epithelial cells can also attenuate CFTR by activating the

autophagosome lysosomal pathway to await the degradation of protein aggregates, further reducing intracellular ROS levels.

42.1.3 P62 and Cystic Fibrosis

CF is an intracellular aggregate-prone disease, and there are a large number of misfolded or modified protein aggregates in CF epithelial cells, such as CFTR, PPAR, and beclin1. P62 is a classical receptor for autophagy, and its protein structure determines its easy formation of aggregates. When the autophagy activity of the cells is impaired, p62 will be accumulated in cells and promotes the onset of CF. After the accumulation of p62, the autophagy activity was inhibited, which led to the dysfunction of the proteasome pathway. A large number of proteins to be degraded in the cells accumulated and could not be removed. Thus, after the autophagy activity of CF epithelial cells was inhibited, the accumulation of p62 also affected the degradation of CFTR mutants to some extent. It has been shown that inhibition of CFTR function in normal bronchial epithelial cells leads to its own ubiquitination, and polyubiquitinated CFTR can interact with the p62 and form aggregates within the cells (Luciani et al. 2010). Moreover, it has been found that internalized CFTR and p62 colocalization in endosomal vesicles. Knockdown of p62 in CF epithelial cells with impaired autophagy activity not only reduces the accumulation of protein aggregates but also promotes the maturation and transport of CFTR proteins. This suggests that restoring cellular autophagy can promote sufficient functional CFTR (Luciani et al. 2011).

42.2 Pulmonary Hypertension and Autophagy

42.2.1 Drugs that Reduce the Death of Pulmonary Hypertension Remain to be Discovered

Pulmonary hypertension is a fatal pulmonary vascular disease characterized by a continuous increase in pulmonary vascular pressure (quiescent pulmonary artery pressure >25 mm Hg) leading to right heart failure even death. A large number of studies have shown that pulmonary hypertension is a reconstituted disease of pulmonary vascular tissue caused by a combination of increased proliferation of vascular smooth muscle cells and decreased apoptosis. Abnormal pulmonary artery remodeling causes vascular obstruction leading to cardiac hypertrophy and cardiac fibrosis; persistent cardiac tissue remodeling leads to right heart failure, left heart failure, and death. Therefore, continuous heart failure is the main cause of disability and death in patients with pulmonary hypertension. Vascular tissue hypoxia caused by various causes is a key mechanism that stimulates vascular smooth muscle cell proliferation

and inhibits cell death, leading to pulmonary hypertension. Although there are currently relatively specific therapeutic drugs for pulmonary hypertension, including endothelial cell receptor antagonists, phosphodiesterase inhibitors, and prostacyclin and analogs, the disease is progressive even in the presence of these specific treatments, because these treatments can't reduce the mortality of patients. Therefore, it is still important to study the pathogenesis of pulmonary hypertension and discover a new strategy for pulmonary hypertension treatment. Recent studies have shown that lung tissue from patients with pulmonary hypertension have increased autophagy activity, suggesting that autophagy may be involved in the development of pulmonary hypertension.

42.2.2 Pulmonary Hypertension and Autophagy

Studies on the pathogenesis of autophagy involved in pulmonary hypertension have just started. Evidence currently shows that activated autophagy may provide protection against the occurrence of pulmonary hypertension, and there is evidence to suggest that inhibition of autophagy may prevent the development of pulmonary hypertension (Nussenzweig et al. 2015). The number of autophagosomes formed in the lung cells is increased in pulmonary hypertension mice induced by chronic hypoxia, and the increase in high pressure occurs in chronic hypoxia which leads to the decreasing of autophagy activity in LC3B-deficient mouse (Rawat et al. 2014). In addition, inhibiting mTORC1, an autophagy negative regulator, activates autophagy to prevent pulmonary vascular smooth muscle cell proliferation. These studies demonstrate that activation of autophagy or inhibition of the mTOR signaling pathway can prohibit the development of pulmonary hypertension. Studies have found that knockout of autophagy signaling molecule beclin1 leads to a lack of autophagy activity, which inhibits the proliferation of pulmonary artery endothelial cells. It has also been found that hypoxia causes an increase in angiogenesis in the beclin1$^{-/+}$ mice. These results demonstrate that beclin1 mediated autophagy is involved in the development of pulmonary hypertension. On the other hand, studies have also provided evidence that autophagy activity promotes the development of pulmonary hypertension (Chen et al. 2018). Indeed, the autophagy-lysosomal inhibitor chloroquine prevents the progression of lung hypertension in experimental animals. Interestingly, recent studies have shown that inhibition of glucose-6-phosphate dehydrogenase in animals with pulmonary hypertension and reduction of nicotinamide adenine dinucleotide phosphate production can inhibit the development of lung hypertension in animals, and improve the heart function. Obviously, studies on the pathogenesis of autophagy and pulmonary hypertension have just begun. The contradictory findings shown above indicate that the role and mechanism of autophagy in the pathogenesis of pulmonary hypertension is unclear.

42.3 Autophagy and Lung Infectious Diseases

42.3.1 Tuberculosis is a Chronic Infectious Lung Disease

Pulmonary tuberculosis, commonly known as pulmonary consumption, is a chronic infectious disease caused by *Mycobacterium tuberculosis* infection in human lungs and is the most common type of tuberculosis in human body. Typical pulmonary tuberculosis is slow, and the course of the disease is long. Common symptoms include low fever, fatigue, loss of appetite, cough, and hemoptysis. However, most patients who suffer from pulmonary tuberculosis often have no obvious symptoms, so that only when examined with X-ray or checked for hemoptysis, patients realize that they got tuberculosis. If tuberculosis can be discovered in time and treated through convincing strategy, most of the patients can be cured. The extent of tuberculosis symptoms is related to the extent of the lesion, progression, and body reactivity. *M. tuberculosis* belongs to the actinomycete family, and its pathogenic effect depends on the inflammatory reaction induced by the proliferation of bacteria in the cells. It also can induce the damage due to promoting the immunoreaction. The infection rate of *M. tuberculosis* is high, but the incidence of tuberculosis is very low, which indicates that the human body has certain immunity against tuberculosis infection. Autophagy is one of the important biological mechanisms of human cells. In addition to regulating cell energy balance, autophagy has also been shown to be involved in pathogen clearance in vivo. To date, studies on the pathogenesis of tuberculosis have focused on autophagy for the elimination of *M. tuberculosis*.

42.3.2 Immune Signal and Autophagy are Effectors of M. tuberculosis

In addition to being induced by starvation, autophagy is also regulated by innate immune signals and inflammatory cytokines during the immune response. It has been shown that activation of many pattern recognition receptors (PRRs) can induce autophagy. For example, ligands of TLR can induce autophagy by stimulating TRAF6 or enhance the stability of autophagy-related proteins such as Beclin1 and ULK1, and NOD receptors signaling can activate autophagy by increasing the protein stability of autophagy-associated proteins RIPK2 and ULK1 also. In addition, cGAMP produced by stimulation with viruses, mitochondria or bacterial DNA, and 3′-5′ circular double GMP or circular double AMP produced by secreted bacteria can also induce autophagy of cells. There is evidence that IL-1β induced autophagy activation is closely related to its anti-tuberculous mycobacterial effect. MyD88 is an important linker regulated by the signaling of different immune receptors. Interestingly, the MyD88 signaling pathway has been shown to play a key role in early anti-tuberculosis processes, but activation of MyD88 signaling via multiple TLRs does not induce significant resistance to tuberculosis. However, the MyD88-dependent

IL-1 signaling pathway plays a key role in the early stages of anti-tuberculosis. Similar to the biological effects induced by IL-1 Th1-type cytokines can also act as intracellular anti-tuberculosis by activating autophagy (Moraco and Kornfeld 2014). IFN-γ is a typical Th1-type cytokine that has been shown to activate autophagy, whereas the Th2-type cytokines IL-4 and IL-13 are capable of inhibiting IFN-γ induced autophagy by STAT6. In addition, Th2-type cytokines can also inhibit starvation-induced autophagy through the Akt signaling pathway.

Calcitriol, a metabolite of Vitamin D, is an important synergist for IFN-γ activation of autophagy. There is a synergistic effect between calcitriol and IFN-γ in macrophages to induce autophagy activation, which effectively promotes intracellular *M. tuberculosis* clearance. Calcitriol can activate the AMPK signaling pathway through Ca2+, activate the ULK1 phosphorylation cascade, and ultimately induce autophagy by forming autophagy core complexes. IFN-γ induced autophagy in macrophages is dependent on calcitriol, but starvation or rapamycin-induced autophagy does not require the involvement of calcitriol. Thus, the decrease of serum calcitriol and its precursor calcifediol in patients with pulmonary tuberculosis limits the anti-tuberculosis effect of IFN-γ.

42.3.3 The Mechanism of the Anti-tuberculosis Effects of Autophagy in Cells

A large body of evidence indicates that autophagy removes intracellular *M. tuberculosis* by the unique antibacterial properties of autophagosomes (Deretic 2014). Through the analysis of autophagy-related factors, it was found that the autophagy cargo protein p62 is a key initiator of the entire autophagy pathway against *M. tuberculosis*. The p62 protein can deliver specific ribosomes (rpS30) and a large number of ubiquitinated cytoplasmic proteins into autophagosomes, and then a series of acidification decomposition reactions occur, resulting in anti-tuberculosis effects. NDP52, another autophagy receptor protein, is also thought to be involved in macrophage-induced autophagy against tuberculosis in mice. Autophagy activation promotes acidification and maturation of M. tuberculosis phagosomes, which in turn become the organelles that kill mycobacteria. The classic anti-tuberculosis strategy is to inhibit phagosome-lysosomal fusion, thereby inhibiting autophagy. However, current evidence suggests that induction of autophagy activation is far superior to the previous classical strategy. The results show that the main component of anti-tuberculosis antibacterial peptide can stimulate the fusion of lysosomes in tuberculosis host cells. Autophagy can also induce antibacterial effects by capturing and digesting components within cells. Host cells infected with *M. tuberculosis* can form autophagosome with antibacterial properties in the cells after induction of autophagy activation. Such autophagosomes can mediate anti-tuberculosis action after fusion with lysosomes. In some cases, intracellular autophagy degradation products contain antimicrobial peptides and derivatives

thereof that promote the clearance of *M. tuberculosis*. In addition, some *M. tuberculosis* escapes from autophagosomes into the cytoplasm, and autophagy can mediate the clearance of escaped *M. tuberculosis* in host cells (Liu et al. 2018).

There is also a close relationship between autophagy and first-line drugs against tuberculosis (Ryter and Choi 2015). For example, isoniazid and pyrazinamide can exert therapeutic effects by activating autophagy. In the treatment of tuberculosis with isoniazid or pyrazinamide, the *M. tuberculosis* product and the oxidative stress products produced by mitochondria and NADPH can synergistically induce activation of autophagy. In addition, several compounds that induce autophagy activation can also act as anti-tuberculosis, suggesting that inducing autophagy is a promising strategy to treat tuberculosis. When the autophagy activity is inhibited by small molecule inhibitors in cells infected with *M. tuberculosis*, the cells no longer have antibacterial activity.

42.3.4 Autophagy and Pulmonary Inflammation Caused by Acute Infection

Pulmonary inflammation is caused by continuous exposure of the respiratory tract to microorganisms, atmospheric particulate matter, irritants, pollutants, allergens, and pathogens. When stimulated, the lungs produce a range of defense reactions. The first is the epithelial barrier, which secretes a large number of substances including mucin, lysozyme, defensin, and nitric oxide; the second is mucociliary, which play an important role in innate defense mechanism. It will remove harmful particles from the mucus of the respiratory tract. In addition, the cells of the natural immune system have the function of aggregating, encapsulating, and killing microorganisms, including macrophages, dendritic cells, monocytes, neutrophils, eosinophils, Natural killer cells, and mast cells. These cells produce a variety of inflammatory mediators such as ROS and various cytokines such as IL6, TNF-γ, and IL1β.

The pathogen recognition receptors expressed on alveolar macrophages and dendritic cells can recognize pathogen-associated molecular patterns and molecular patterns of damage, and respond to infection or injury of the respiratory tract. Subsequent inflammatory cascades and cytokines further recruit immune cells such as neutrophils and monocytes. In addition, dendritic cells also acted as an antigen-presenting cell to associate innate immunity with acquired immunity by phagocytizing microorganisms and migrating to local lymph nodes to activate lymphocytes including T cells and B cells. During pulmonary infection, alveolar macrophages can regulate inflammation and damage repair processes by killing microbes and clearing apoptotic cells. During the infection, neutrophils are located in the pulmonary capillaries and interstitial spaces, and are recruited into the alveolar cavity during infection by the chemokines. Neutrophils can secrete antibacterial proteins and reactive oxygen species to kill the ingested microorganisms, and simultaneously release chemokines to recruit more monocytes to the site of infection.

The lymphocytes in the lung tissue are distributed throughout the airway and lung parenchyma and consist of thymus-dependent T cells and bone marrow-dependent B cells. The activated Th1 cells can produce pro-inflammatory cytokines including TNF-α and IFN-γ while Th2 cells secrete IL4, IL13 that stimulate B cells to produce immunoglobulin E concentration, Serum (IGES) to activate mast cells. In addition, IL5, another important Th2 cytokine, could stimulate eosinophils and further exacerbate local tissue inflammation. During inflammation, mast cells also produce cytokines, leukotrienes, and proteases by activating IGES receptor (FCER1).

In order to remove pathogens and harmful particles from the lung tissues, the body needs a rapid, powerful, and highly regulated inflammatory defense reaction. Furthermore, the steady state in the respiratory system depends on the collaboration between the innate immune system and the adaptive immune system. Autophagy plays an important role in the inflammatory response of the lungs suffered from infection or stress. At homeostasis, autophagy in alveolar macrophages is essential for the inhibition of spontaneous inflammation, and autophagy is also the basic mechanism for airway goblet cells to secrete airway mucus. $Atg5^{-/-}$ or $Atg7^{-/-}$ deficient mice develop spontaneous aseptic pulmonary inflammation characterized by a marked increase in the number of inflammatory cells, submucosal thickening, goblet cellification, and collagen increase. Knocking out ATG 5 in mouse ITGAX/$CD11c^{+}$ cells results in spontaneous airway hyperresponsiveness and severe neutrophilic lung inflammation.

In acute lung injury, autophagy activation is a mechanism of host protection following bacterial and viral infection. Atg7 knockout mice reduce the ability of pathogen clearance, increase neutrophil-mediated inflammation and IL1β production, leading to severe lung damage and reduced survival after infected with P. aeruginosa. Similarly, map1l3b knockout mice infected with respiratory syncytial virus present severe IL17A-dependent lung pathology change and increase Th2 cytokine levels, mucus secretion, and infiltrating of eosinophils and dendritic cells in the lungs. At the same time, it has been suggested that autophagy appears to be a protective response in infected lungs, and autophagy may play an adverse role in the late stages of sepsis due to excessive autophagosome accumulation, leading to acute lung injury. In addition, in LPS-induced pulmonary inflammation, knockdown of PI3KC3 in macrophages results in loss of autophagy, which reduces the infiltration of immune cells in the bronchi and alveoli of the lungs, and the concentration of cytokines in the lungs. These evidences suggest that autophagy plays a key role in acute lung infections, including clearance of pathogenic microorganisms, the development of inflammatory responses, and the maintenance of lung tissue homeostasis.

42.3.5 Sepsis and Autophagy

Although the immune response is critical for the host to fight against sepsis, excessive immune responses and inflammation often cause tissue/organ damage and subsequent secondary infection. The role of autophagy in sepsis is investigated based

on transmission electron microscopy studies of liver specimens from patients with sepsis. These studies have shown an increase in the number of autophagic vacuoles in the liver of patients with sepsis compared with non-sepsis patients. However, it is unclear whether the increase in autophagic vacuoles in patients with sepsis means an increase in autophagy activity in patients with sepsis, or an inhibition of autophagy leading to excessive accumulation of autophagic vacuoles. However, these data indicate that autophagy plays an important role in the pathogenesis of sepsis. Autophagy has been shown to play an important role in preclinical models of sepsis such as sepsis caused by cecal ligation and perforation (CLP) and endotoxin-induced septic shock. The level of LC3B is up-regulated in target organs of sepsis such as lung, liver, and kidney of mice receiving CLP or endotoxin treatment. The study of autophagy in the sepsis models is based on various transgenic mice or autophagy modulators. Deletion of autophagy genes such as MAP1LC3B, BECN 1, or Vps 34 increases inflammation, bacterial burden, organ damage, and mortality in mice with sepsis caused by CLP or LPS. In contrast, autophagy activators such as rapamycin or overexpressing LC3 inhibit inflammatory and apoptotic activities and increase survival in CLP mice. Genetic studies have further shown that autophagy has important biological functions in sepsis. The immune-related GTPase acts as an important molecule regulating autophagy induction and elimination of intracellular mycobacteria, and its genetic polymorphism is associated with mortality in patients with severe sepsis. Mutations in the ATG 16 allele are also associated with sepsis severity and ventilator-associated pneumonia.

Although modulating inflammation is an important method of preventing multiple organ dysfunction in sepsis, proper immune response is crucial for eliminating microorganisms during infection. In this regard, autophagy can upregulate immune function through allogeneic autophagy and mitochondrial autophagy, increasing bacterial killing and inhibiting inflammation. The killing effect of allogeneic autophagy on virulence pathogens can reduce the number of pathogens, leading to immune reactions and inflammation during sepsis. In addition to allogeneic autophagy, autophagy can also regulate immune responses and inflammation by controlling mitochondrial quality. Increased plasma mitochondrial DNA levels in patients with sepsis are associated with disease severity and mortality. In contrast, a recent study showed that the copy number of mitochondrial DNA in monocytes and lymphocytes in patients with sepsis is reduced and inversely associated with the severity of the disease, which suggested that mitochondrial DNA can be released from these cells. The release of mitochondrial DNA is associated with mitochondrial DNA-mediated activation of NLRP 3 inflammatory bodies. Therefore, these results indicate that mitochondrial integrity may be impaired in patients with sepsis.

Mitochondrial dysfunction promotes ROS production in mitochondrial and activation of NLRP 3 inflammatory bodies, leading to cell death and secretion of proinflammatory cytokines. Mitochondrial dysfunction caused by infection or oxidative stress during sepsis may be one of the leading causes of immune response. Therefore, the elimination of dysfunctional mitochondria by mitochondrial autophagy is critical for regulating immune responses and inflammation. LC3B is increased and co-localized with mitochondria in the lungs of mice with sepsis caused by

S. aureus infection, indicating that mitochondrial autophagy has changed after infection. Indeed, the deletion of LC3B and Beclin 1 further increased the production of IL-1β and IL-18 in septic mice and mortality. In addition, the lack of Parkin gene impaired myocardial contractility recovery in LPS-treated septic mice. Defects in mitochondrial autophagy caused by kinase JNK 2 deficient mice also cause excessive activation of inflammasome and increase mortality due to endotoxic shock.

The immunomodulatory effects of autophagy may be related to changes in cellular metabolism. Recent studies have shown that downregulation of cellular metabolic pathways, including glycolysis, lipid synthesis, and fatty acid synthesis, is critical for the activation of inflammasome. Hexokinase-1 (HK-1), uncoupling protein-2 (UCP2), and NADPH oxidase 4 (NOX4) are known key molecules for this process. Although the important role of autophagy in immune cells such as macrophages has been confirmed, it is unclear whether other types of cells are involved in the pathogenesis of sepsis. Given the complexity of sepsis pathology, further studies using genetic and other approaches are needed to determine the role of autophagy in various cell types/tissues.

42.4 Obstructive Sleep Apnea (OSA) and Autophagy

Obstructive sleep apnea (OSA) is a respiratory disorder that occurs during sleep, with recurrent upper airway obstruction leading to apnea, hypopnea, and/or respiratory arousal. These respiratory disorders often lead to sleep disruption, hypoxemia, hypercapnia, and increased sympathetic activity, while increasing the risk of hypertension, congestive heart failure, type 2 diabetes, stroke, and premature death. This is an area of increasing public health that is of concern. The latest data show that the prevalence of moderate to severe OSA in men is 10–17%, and in women it is 3–9%. Compared with the past 20 years, the prevalence rate has increased by more than 25%. The underlying pathological mechanisms leading to systemic dysfunction in OSA patients are complex and not fully understood, but it has been illustrated that this effect is associated with intermittent hypoxia (IH) in patients. The relationship between intermittent hypoxia and abnormal glucose metabolism and obesity has been established by in vivo experiments. However, there is increasing evidence that IH plays an important role in causing inflammation, which induces OSA-associated cardiac metabolic diseases. IH is a known activator of the NF-κB 1/2 pathway, which acts as a potent inflammatory activating factor leading to the release of TNF-α, IL6, IL8, and CCL 2/MCP-1. Patient data indicate that increased circulating ROS in OSA patients, but the evidence is limited. Although this issue needs further study, systemic inflammation does have an important role in the pathogenesis of OSA-related diseases.

In recent years, the role of autophagy in the pathogenesis of OSA has been questioned. In vivo studies have linked autophagy to chronic intermittent hypoxia. Using a chronic intermittent hypoxic rat model, activating autophagy by injecting melatonin protects the body from heart damage, which is a common cause of OSA disease.

Interestingly, another study tested the role of autophagy in insulin resistance caused by chronic intermittent hypoxia, and there is no relationship between these processes. These data suggest that autophagy may play a role in the processes of chronic intermittent hypoxia in OSA patients. However, more research is needed to determine which processes are dependent on the regulation of autophagy. As an important research field, using autophagy activators/inhibitors may be an important approach to modulate systemic complications of OSA disease.

42.5 Conclusion

Respiratory diseases are usually chronic disorders that have long threat to public health and life. The constant exploration of the pathogenesis of these diseases will provide new information to discover new therapy strategies and drugs. Autophagy, as one of the important biological regulatory mechanisms of cells, is involved in the occurrence and development of many respiratory diseases, especially considering that the basal autophagy activity in cells is a necessary condition for the resolution of various respiratory diseases. Indeed, autophagy is an important mechanism for repairing lung tissue damage, regardless of the cause of lung tissue damage. However, chronic inflammation in the damaged lung tissue will block the autophagy process and decreases autophagy activity. Neither the over-activated autophagy nor the blocked autophagic flux can trigger tissue repair and lung regeneration, and both of these processes cause lung injury by inducing pro-inflammatory and pro-apoptotic effects. Therefore, understanding the role of autophagy in the development of various respiratory diseases is important for the therapy of a variety of lung diseases, especially chronic respiratory diseases.

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Part X
Autophagy and Malignant Hematological Diseases

Autophagy plays a very important role in the differentiation and maturation of hematopoietic stem cells. It is a specific mechanism for reticulocytes to clear mitochondria. Studies on lymphocyte differentiation found that mice lacking Atg5 (autophagy-associated gene 5) have significantly reduced numbers of peripheral blood T cells, B lymphocytes, and thymocytes, while circulating $Atg5^{-/-}$ thymocyte death is significantly increased. Mammalian target of rapamycin (mTOR) is a key regulator of autophagy pathways, including mTORC1 and mTORC2, in which mTORC1 protects megakaryocytes from autophagic death and inhibits mTORC1-induced megakaryocyte autophagy, subsequently blocking the terminal differentiation of megakaryocytes (Kim and Guan 2015).

Autophagy plays a key role in tumor development. The process of regulating autophagy in tumor cells is extremely complicated. On the one hand, it inhibits the growth of tumor cells, on the other hand, it promotes the survival of tumor cells under certain stress conditions. Beclin1 is one of the most important positive regulators of autophagy. Beclin1 deficiency is present in a variety of solid tumors, such as breast cancer, ovarian cancer, and prostate cancer, suggesting that autophagy may cause tumorigenesis, and autophagy assists tumor cells in coping with nutrient deficiencies and hypoxia, scavenging oxygen-free radicals and combatting mitochondrial damage, among others, thereby promoting tumor cell growth (Auberger and Puissant 2017).

Autophagy plays an extremely important role in the development and treatment of hematological malignancies (Nencioni et al. 2013). We will provide a detailed description of the roles/applications of autophagy in the pathogenesis and treatment of leukemia, lymphoma, and myeloma.

Chapter 43
Autophagy and Leukemia

Zhong Zheng, Li Wang, Shu Cheng, Yan Wang, and Weili Zhao

Abstract Leukemia is a malignant clonal disease that originates from hematopoietic stem cells. As in-depth research examines the molecular biology and immunology of the hematopoietic system, leukemia treatment has evolved from a single cytotoxic drug to treatments that inducing differentiation and apoptosis. Meanwhile, autophagy has become a growing concern as a new form of cell death. The immune response, hematopoietic stem cell differentiation, and drug resistance of tumor cells are all potentially affected by autophagy. Regulating autophagy may become one of the promising directions in the field of targeted therapy.

Keywords Autophagy · Acute myeloid leukemia · Myelodysplastic syndrome · Chronic myeloid leukemia

43.1 Autophagy and Normal Hematopoietic Cell Differentiation

Autophagy is recognized as an important form of programmed cell death, involved in the differentiation of hematopoietic cells, including differentiation of reticulocytes into mature red blood cells, formation of preplatelets, differentiation and maturation of myeloid and lymphoid cells (Garcia-Prat et al. 2016).

When abundant nutrients are available, autophagy is mainly used to remove excess metabolites, damaged organelles, and protein aggregates from cells. The process of the selective clearance of mitochondria by autophagy is called mitochondrial phagocytosis (mitophagy). Mitochondrial phagocytosis plays a crucial role in the development of the erythroid system. In mammals, precursor cells derived from hematopoietic stem cells develop into normoblasts in the bone marrow and then denucleate to form reticulocytes. The newly regenerated reticulocytes enter the peripheral circulation and further develop into mature red blood cells, forming a double-sided

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concave shape and removing residual organelles (mainly mitochondria). In this process, mitochondrial phagocytosis is the main mitochondrial degradation pathway. During retinoblast maturation, erythropoietin upregulates BNIP3-L (BCL2 interacting protein 3), a selective autophagy receptor binds to the LC3 protein and mediates mitochondrial phagocytosis. BNIP3-L-deficient mice show a compensatory increase in the number of erythroid precursor cells and a decrease in the number of mature red blood cells, suggesting that BNIP3-L is an important regulator of erythropoiesis and maintains blood homeostasis. P62/SQSTM1 is also involved in the clearance of mitochondria and ribosomes in the terminal phase of erythroid differentiation (Sbardella et al. 2017). In addition, the process by which Atg12 binds to Atg5 and LC3 to form autophagosomes requires the involvement of Atg7, while the mitochondria in reticulocytes are not cleared in Atg7-deficient models. Mice with hematopoietic cells lacking Atg7 develop severe anemia that may be associated with mitochondrial clearance disorders. Based on these phenomena, autophagy plays a crucial role in the differentiation and maturation of the erythroid terminal phase.

The key mediator of autophagy, mTOR, regulates the proliferation of megakaryocyte precursor cells and induces the terminal differentiation of megakaryocytes. According to a previous study, mTORC1 protects megakaryocytes from autophagic death and mTORC2 affects megakaryocyte production by regulating the cell cycle. Thus, a certain correlation exists between the mTOR pathway and megakaryocyte differentiation.

Autophagy regulates the proliferation and differentiation of B and T lymphocytes. The role of autophagy in the development and survival of T lymphocytes was first discovered in Atg5-deficient mice. The total number of thymocytes and the numbers of peripheral T and B lymphocytes were significantly reduced in $Atg5^{-/-}$ mice. $Atg5^{-/-}$ thymocyte death in the circulation increased, and the proliferative activity was also altered after binding to the T cell receptor. Based on above results, autophagy affects T lymphocyte proliferation, while its effect on differentiation is limited. A genetic analysis of Atg5 expression revealed that the Atg5 is involved in mitochondrial autophagic clearance when expressed at high levels (O'Sullivan et al. 2016).

In addition, autophagy is an important component of nonspecific immune responses that are capable of clearing pathogens within cells. Autophagy prevents anti-inflammatory mechanisms, regulates the homeostasis of the body and prevents tissue damage. Recent studies suggest that autophagy may be related to Toll-like receptors (TLRs) (Deretic and Levine 2018).

43.2 Autophagy Promotes Leukemia Cell Death

Mitochondrial dysfunction results in the autophagic death of the cells. Some triterpenoid derivatives exert toxic effects on the mitochondria of chronic myeloid leukemia (CML) cells and cause autophagic death; these compounds are expected

to be a new class of antitumor drugs. The anti-tumor effects of certain quinoline-like drugs are also associated with the activation of autophagy. Thus, mitochondrial damage often coincides with autophagy initiation. Autophagy plays an important role in the death of leukemia cells, and many drugs can induce autophagy. Various compounds have been found to activate autophagy and thereby exert an anti-leukemia effect. In a murine model, bortezomib stimulated the conversion from LC3-I to LC3-II, inducing autophagy while decreasing the level of FLT3-ITD protein in vivo to improve overall survival. The mechanism underlying the effects of bortezomib is related to the inhibition of MAPK/ERK pathway, PI3K/AKT pathway and STAT5 molecule, which in turn activates autophagic death. In the treatment of myeloid leukemia, vitamin D upregulates the expression of the beclin1 gene, which in turn binds to phosphatidylinositol 3 kinase and initiates autophagy, leading to the autophagic death of leukemia cells. Dexamethasone also induces autophagic death in acute lymphocytic leukemia (ALL) cells. Acadesine initiates autophagy by activating PKC. Low-dose GX15-070 monotherapy inhibits cell growth and induces BAK-dependent apoptosis and Atg5-dependent autophagy. Resveratrol (RSV) induces the expression of p62/QSTM1 via the JNK pathway, activates the AMPK pathway and promotes autophagy in CML cells. Arsenic trioxide induces autophagy in leukemia cells, and autophagy inhibitor 3-MA inhibits arsenic-induced autophagy, enhancing the anti-leukemia effect. Although these drugs have different mechanisms of action, they all achieve their anti-leukemia effects by inducing autophagic death through various autophagy-related signaling pathways.

Epigenetic changes are also important for inducing autophagic death in cells. Continuous exposure of myeloid leukemia cells to the demethylating drug decitabine promotes the differentiation of erythroid and megakaryocytic cells, while promoting autophagy and increasing leukemia cell death. A genome-wide analysis revealed an association between the degree of methylation of autophagy-related genes in CML cells and a poor prognosis, while a blockade of autophagy reduced leukemia cell clearance. Bcl-2 antagonists activate autophagy-dependent necroptosis, reverses mitochondria-dependent apoptosis, restores the susceptibility to glucocorticoids and cytotoxic drugs. Therefore, strategies that regulate autophagy may be one way to reverse multidrug resistant leukemia (Djavaheri-Mergny et al. 2019).

43.3 Autophagy Maintains Leukemia Cell Survival

Autophagy is induced to produce nutrients and energy under starvation or stress, promoting survival. It is important for the survival of hematopoietic stem cells. Mitochondrial damage and oxygen free radicals (ROS) accumulate in autophagy-deficient hematopoietic precursor cells and induce an increased rate of cell proliferation and apoptosis in an Atg7-deficient mouse model. Above phenomenon is frequently observed in the patients cells with myelodysplasia syndrome (MDS). Autophagy-deficient mice usually die within 12 weeks and primordial myeloid cells infiltrate multiple organs including the liver, spleen, heart, small intestine and other

organs. In addition to Atg7, the Ulk1 autophagy promoter complex FIP200 is also a target that has been examined in recent studies. FIP200 knockout mice exhibit an accumulation of mitochondria and elevated levels of oxygen free radicals, resulting in severe anemia, hematopoietic stem cell components loss and abnormal proliferation.

Activation of autophagy might enhance the resistance of leukemia cells to chemotherapy, and inhibition of autophagy is expected to increase the sensitivity to chemotherapy. Studies on multiple leukemia cell lines have shown a significant increase in the number of intracellular autophagy bodies after imatinib treatment. Imatinib increases the expression of the beclin1 and Atg5 genes by inhibiting c-Abl, subsequently promoting autophagy and protecting cells. Autophagy is increased in CML cells expressing Bcr-Abl at high levels, which helps cells to antagonize the therapeutic effects of tyrosine kinase inhibitors (TKIs). Another study reported the inhibition of autophagy enhanced the therapeutic effects of TKIs on Ph-positive hematological tumors and restored the sensitivity of CML stem cells to TKIs.

Cells receiving chemotherapy produce specific damage-related molecular patterns (DAMPs) that activate autophagy pathways and induce the development of drug resistance in leukemia cells. Malignant melanoma differentiation-related gene-7/interleukin-24 (mda-7/IL-24) contributes to leukemia cell survival by inducing autophagy. Autophagy protects cells treated with phototherapy (PDT). An increase in the PDT dose induces cell death (Moosavi et al. 2016). The cytoprotective effect of autophagy may be one of the mechanisms leading to chemotherapy resistance. Autophagy will provide new therapeutic prospects for reversing drug resistance in clinical practice.

Currently, targeted autophagy has become a potentially novel treatment for hematological malignancies. Most chemotherapy drugs induce both apoptosis and autophagy. Although apoptosis and autophagy are two different cell death pathways, they also engage in cross-talk. Caspase-8 in the apoptotic pathway regulates autophagic death, and Caspase-8 inhibitors promote autophagy by regulating beclin-1 and Atg7. The bcl-2 family and Tp53 in the apoptotic pathway also play an important role in the autophagy pathway. The addition of sodium selenite to acute promyelocytic leukemia (APL) cells decreases autophagy and promotes apoptosis. The AKT inhibitor triciribine increases apoptosis in T-ALL cells, probably because autophagy becomes a cytoprotective mechanism when apoptosis is increased. Once autophagy is inhibited, cell apoptosis will increase. In fact, autophagy and apoptosis often coexist during cell death. Hematopoietic tumor cells that are insensitive to apoptosis-inducing agents due to changes in the apoptotic pathway may also be highly sensitive to autophagy inducers, therefore, the use of strategies targeting autophagy to treat hematopoietic tumors has received increasing attention (Djavaheri-Mergny et al. 2019).

43.4 Autophagy in Various Types of Leukemia

43.4.1 Myelodysplastic Syndrome (MDS)

MDS is a clonal disease of hematopoietic stem cells. In patients with low-risk MDS, megakaryocytes die prematurely due to apoptosis, autophagy or necrosis, which is the main cause of thrombocytopenia. A red blood cell maturation disorder and mitochondrial dysfunction are major features of low-risk MDS. Patients with refractory anemia (RA) or refractory anemia with ring-shaped iron granulocytes (RARS) and erythrocytes often display autophagy deregulation.

Abnormal changes in the mitochondrial structure are observed in individuals with MDS, and the mitochondria are enlarged in primitive nucleated red blood cells, basophilic nucleated red blood cells and more mature nucleated red blood cells. Since the mitochondria are the main regulators of the caspase-dependent apoptotic pathway, apoptosis is increased in individuals with MDS. Anemia is caused by an imbalance between hematopoietic stem cell proliferation and apoptosis. Atg7-deficient mouse erythrocytes and hematopoietic precursor cells are more sensitive to caspase-3-dependent apoptosis (Wu et al. 2014). Abnormal mitochondrial function increases the levels of oxygen free radicals. In addition, mitochondrial iron overload is a common phenomenon observed in patients with MDS, particularly patients with the RARS subtype. Excess iron deposition is also an important factor that promotes the formation of oxygen free radicals. Excess levels of oxygen free radicals may inhibit the activity of tumor suppressor genes such as PTEN, promote autophagy, and thereby eliminate intracellular senescence and damaged mitochondria (Filomeni et al. 2015). Mitochondrial phagocytosis, a process associated with autophagy, is not only involved in cell damage and apoptosis but also plays an important role in the transformation of MDS to AML. The number of mitochondrial autophagosomes is significantly increased in the hematopoietic precursor cells of patients with low-risk MDS. When mitochondrial autophagy is defective, mitochondria are not normally degraded and produce a large amount of oxygen free radicals, causing DNA damage. If these abnormal mitochondria are capable of releasing a sufficient amount of cytochrome C to cause apoptosis, sustained DNA damage will promote the conversion of MDS to AML. The presence of a large number of autophagosomes loaded with mitochondria in patients with low-risk MDS indicate that the autophagy function is basically normal, which may be one of the reasons for the low AML conversion rate in low-risk patients. Therefore, to some extent, autophagy prevents the conversion of MDS to AML (Jiang et al. 2018).

Although autophagy/mitochondrial phagocytosis is associated with the pathogenesis of MDS, no clear changes in autophagy-related genes have been detected in patients with MDS to date, which is partly related to the strong heterogeneity of MDS. Different subtypes may have different gene expression profiles that do not completely reflect the functional point mutations in the coding regions of autophagy/mitochondrial phagocytosis-related genes. Researchers have not clearly

determined whether autophagy is regulated at the transcriptional level or whether changes in transcription levels reflect altered autophagy activity.

43.4.2 AML Is a Cancer of the Myeloid Line of Blood Cells

In patients with AML presenting with complex karyotypes, the expression of autophagy-related genes Atg10, Atg12, Atg9B, PRKAG2, KLHDC10, GABARAPL2, MAP1LC3B, and GABARAP (the latter three are homologues of human Atg8) is proportionally increased. Autophagy regulates HSC proliferation and differentiation by activating downstream signaling pathways, such as the Notch signaling pathway. Decreased autophagy induces normal hematopoietic stem cells to become preleukemia cells, and a heterozygous ATG5 deletion enhances tumor cell invasiveness in AML mouse models (Watson et al. 2015). In addition, hypermethylation of the autophagy-related gene Atg16L2 is observed in leukemias and lymphomas. An allelic deletion of beclin1 was detected in leukemia and other solid tumors, such as breast, ovarian, and prostate cancer (Ishibashi et al. 2011). Based on these data, autophagy protects normal cells with stable cellular genetic material and may inhibit tumor development. Changes in the expression of autophagy-related genes alter the development of leukemia.

Activation of the PI3K/AKT/mTOR pathway in AML is closely related to autophagy in cells. Low doses cytarabine (Ara-c) significantly inhibit the growth of the AML cell line U937 and HEL cell lines in a time- and dose-dependent manner. After treatment with low dose Ara-C (50 nM), the expression of LC3 and beclin1 in U937 and HEL cells increased over time, and the degradation of p62 increased. Characteristic autophagosomes appeared 24 h in vitro treatment. At the same time, the activation of the AKT-mTOR pathway was inhibited (Chen et al. 2017). All trans retinoic acid (ATRA), alone or in combination with arsenic trioxide (ATO), has been shown to induce the differentiation of APL cells. ATRA and ATO mediate the degradation of the PML-RARA oncoprotein through the mTOR-related autophagy pathway, regulate the cationic glycerol-6-phosphate receptor and promote autophagosome maturation and sac acidification. HMGB1 (high mobility group box 1) was recently shown to play an important role in the development of autophagy. HMGB1 is transferred from the nucleus to the cytoplasm, regulating the binding of p62/SQSTM1 and PML-RARA to mediate PML-RARA degradation (Auberger and Puissant 2017). The key enzyme involved in NAD biosynthesis, nicotine phosphoryl acyltransferase inhibitor APO866, exerts strong cytotoxic effects on hematological cell lines, including AML, ALL, CLL, CML, T-cell lymphoma and mantle cell lymphoma lines. However, it is not toxic to normal hematopoietic precursor cells. Treatment with APO866 reduces NAD and ATP levels and promotes cell death through a mechanism that does not depend on the caspase pathway but associated with mitochondrial dysfunction and the induction of autophagy. APO866 inhibits tumor growth in a mouse model of human leukemia without significant toxicity. Similarly, an Arabic tea extract also causes autophagy in AML cells by damaging

mitochondria. Thus, autophagy may become a potential therapeutic target for various hematological tumors.

The role of autophagy in the development of AML has not been conclusively determined. Compared with normal hematopoietic cells, the level of autophagy in AML cells is low and mutations in autophagy-associated proteins in primary cells cause a loss of function. Therefore, autophagic defects caused by mutations may be involved in the initiation and progression of AML. In contrast, the key autophagy protein ATG7 has been identified as a key resistance factor affecting AML. Although these studies are very meaningful, they do not address the relationship between the heterogeneity of AML subtypes and the diversity of autophagy states. The FLT3-ITD subtype is observed in 25% of patients with AML. FLT3-ITD expression increases autophagy in AML cells, whereas inhibition of FLT3-ITD activity decreases autophagy levels in primary AML cells and AML cell lines. The conditional shRNA-mediated knockdown of key autophagy proteins indicates that autophagy is required for the proliferation of AML cells in vitro and survival of xenografted mouse leukemia cells in vivo. The inhibition of autophagy also allows cells to overcome resistance to FLT3 inhibitors in vitro and in vivo. The transcription factor ATF4 is considered an important factor involved in FLT3-ITD-induced autophagy. The cellular level of ATF4 is highly dependent on FLT3-ITD activity and downregulation of ATF4 inhibits the proliferation of AML cells and improves the overall survival of mice. Based on these results, approaches targeting autophagy may represent an innovative AML treatment strategy in patients with FLT3 mutations (Heydt et al. 2018).

The characteristic APL fusion gene PML/RARα is a substrate for autophagy, and autophagy activation contributes to the treatment of APL. PML/RARα enhances autophagy by inhibiting the Akt/mTOR signaling pathway. An ATRA treatment of APL is capable of inducing autophagy and promoting the degradation of the PML/RARα fusion protein. The ubiquitin proteasome system is essential for the degradation of PML/RARα. Autophagy-associated FYVE domain-containing protein ALFY/WDFY3 is involved in the autophagy-mediated degradation of protein aggregates, thereby promoting autophagy induced by ATRA therapy. Levels of the ALFY mRNA are significantly increased in AML cells treated with ATRA. In addition, ALFY depletion impairs ATRA-induced granulocyte differentiation. ALFY knockout results in a decrease in ATAR-induced proteolysis, indicating that ALFY plays a crucial role in the autophagy-mediated degradation of PML-RARα and in the retinoic acid-induced AML cell maturation process (Schlafli et al. 2017). Another key drug for the treatment of APL, ATO, is also capable of inducing cell death by activating autophagy in APL cells. This effect has been confirmed in experiments with the autophagy inhibitor chloroquine and the knockout of key molecules such as beclin1 and Atg7. The PLZF/RARα fusion gene produced by the t(11;17) translocation represents another special subtype of APL that responds poorly to both ATRA and ATO. Researchers have not clearly determined whether activated autophagy clears PLZF/RARα. In normal hematopoiesis, ATRA not only induces the differentiation of myeloid cells but also promotes the self-renewal of hematopoietic stem cells and precursor cells. In addition to APL cells, ATRA also induces the proliferation and/or self-renewal of leukemia stem cells, which may be a possible cause of

APL molecular remission and recurrence after treatment with ATRA monotherapy (Dos Santos et al. 2013).

The AML1-ETO fusion gene is frequently observed in AML differs from PML/RARα. AML1-ETO is a fusion oncoprotein produced by t(8;21) that triggers AML in combination with mutations in genes such as c-Kit, ASXL1/2, FLT3, N-RAS and K-RAS. Caspase-3 is one of the key executioner proteins. Caspase-3 directly degrades AML1-ETO in vitro, suggesting that AML1-ETO may accumulate in cells lacking Caspase-3 activity, thus accelerating the occurrence of leukemia. A Caspase-3 knockout mouse model was established, and the loss of Caspase-3 delayed the initiation of AML1-ETO9a leukemia, suggesting that Caspase-3 may play different roles in the initiation and progression of AML. The absence of Caspase-3 triggers a conserved ND adaptive mechanism, by which autophagy ultimately limits AML1-ETO9a-driven leukemia. ULK1 is a novel substrate for Caspase-3. Upregulation of ULK1 drives the initiation of autophagy in leukemia cells. Inhibition of ULK1 rescues the phenotype caused by Caspase-3 deletion in vivo and in vitro. Based on these findings, Caspase-3 is an important factor regulating autophagy in AML. Due to the presence of transcriptional repressor complexes, the normal differentiation of primordial cells is blocked. These transcriptional repressor complexes contain large amounts of histone deacetylase, making them sensitive to histone deacetylase inhibitors (HDACIs). HDACIs can induce autophagy, among which the most representative drugs are sodium valproate and vorinostat (Auberger and Puissant 2017). Inhibition of autophagy by chloroquine in AML1-ETO-positive cells and patient samples significantly increases HDACI-induced cell death. After knock out of the autophagy core components Atg7 and ULK1, autophagy exerts a protective effect on leukemia cells. HDACI-induced autophagy promotes leukemia cell survival through several pathways. First, HDACIs increase ubiquitin levels in proteins, which are further elevated upon the inhibition of autophagy, suggesting that autophagy prevents HDACI-induced protein accumulation. Second, autophagy participates in redox reactions by removing damaged organelles such as mitochondria. The combination of the active oxygen scavenger N-acetylcysteine with autophagy inhibitor blocks HDACI-induced cell death (Altman et al. 2014). In addition, HDACIs overacetylate heat shock protein (HPS90) to decrease its activity. Many genes that affect the growth of leukemia cells (such as AKT1, Kit/c-Kit, and BCR-ABL) interact with HPS90. Previous studies have confirmed that sodium valproate and vorinostat rapidly downregulate Kit and its downstream signaling molecules. In addition, HDACIs also affect the activity of STAT3 and mTOR by inhibiting the mTOR, Akt1 or MAPK pathway through the consumption of growth factors. Therefore, HDACIs activate autophagy by preventing the deacetylation of autophagy core components (Baek and Kim 2017). Some fusion proteins are selectively degraded by autophagy, probably because autophagy is mainly directed to remove protein aggregates, and the aggregation of proteins determines the degradation pathway. PML/RARα forms large aggregates and is only effectively dissolved in 8 M urea. Similarly, BCR/ABL is also a highly aggregated fusion protein. The clearance of PML/RARα and BCR/ABL requires the autophagy receptor SQSTM1/p62 that binds to ubiquitin. In contrast, AML1-ETO is a relatively low-concentration fusion protein that is easily dissolved

in RIPA buffer, and thus is not an ideal substrate for autophagy. An understanding of the role of autophagy in different subtypes of leukemias expressing various specific fusion proteins and the different mechanisms of action will effectively guide clinical treatment.

In other AML cell lines that do not have a well-defined oncogene expression profile, autophagy also causes leukemia cell death. Notably, 1,25-dihydroxyvitamin D3 has also been used as an active form of vitamin D3 to treat leukemia. The separation of vitamin D3 from Raf1 activates the Raf/MEK/ERK2/MAPK pathway and inhibits the proliferation of tumor cells by inducing differentiation. Vitamin D3 activates autophagy by upregulating beclin1 in myeloid leukemia cells. Knockout of beclin1 not only affects vitamin D3-induced autophagy but also inhibits cell differentiation and apoptosis, indicating that intracellular autophagy is inextricably linked to cell differentiation and apoptosis (Mushegian 2017).

43.4.3 In Acute Lymphoblastic Leukemia (ALL), Low Concentrations of Bafilomycin A1 Effectively Inhibit B-ALL Cells in Children with Acute Lymphoblastic Leukemia

Based on the results from in vitro and in vivo models, bafilomycin A1 downregulates cytoprotective autophagy in a mouse model, inhibits primary leukemia cell proliferation and delays leukemia by inducing apoptosis. In vivo toxicity experiments further confirmed the safety of bafilomycin A1. Therefore, bafilomycin A1 has the potential to become a novel therapeutic drug for children with B-ALL. Using the t(1;19) mouse model as an example, prophylactic use of rapamycin restores the function of hematopoietic stem/progenitor cells by activating autophagy and improves the survival rate of mouse models with leukemia. Leukemia cells showed cyclical arrest in mice treated with rapamycin. Autophagy is activated by rapamycin or starvation to degrade the oncogenic protein E2A/Pbx1. In addition, E2A/Pbx1 and the autophagy marker LC3 colocalize in autophagosomes and induce autophagy through a ubiquitin-mediated pathway. These studies further confirm that autophagy plays an important role in mediating the degradation of E2A/Pbx1 oncoproteins. Glucocorticoids are widely used to treat lymphoid tumors and induce apoptosis. In ALL, the inhibition of autophagy with a siRNA targeting the beclin1 gene interferes with dexamethasone-mediated apoptosis. The induction of autophagy by dexamethasone depends on high levels of PML protein, which in turn causes Akt inactivation. Glucocorticoid resistance often suggests a poor prognosis. Everolimus (RAD001) is an inhibitor of mTORC1 that promotes apoptosis and autophagy in ALL cells. The effect of everolimus on apoptosis is limited, but it increases the expression of beclin1 and induces autophagy to exert an anti-leukemia effect. Children with ALL, everolimus significantly reduces tumor volume and improves survival by targeting the AMPK/mTOR signaling pathway (Baraz et al. 2014).

43.4.4 Chronic Myeloid Leukemia (CML) Is a Myeloproliferative Disease Characterized by a Marked Increase in Neutrophils in the Peripheral Blood and Bone Marrow, Accompanied by Significant Splenomegaly

CML is characterized by a chromosomal translocation at t(9;22) (q34;q11) that produces the BCR-ABL fusion gene and continuously activates the tyrosine kinase. BCR-ABL expression causes the hyperproliferation of hematopoietic stem cells, which is related to PI3K/Akt/mTOR and ERK signaling pathways that inhibit autophagy. PI3K/Akt is a downstream gene of BCR-ABL that induces mTORC1 activation and inhibits autophagy. Therefore, the ability to restore autophagy activity has become one focus of CML treatment. In addition, ATG7 or ATG4b knockdown may alter the viability of CD34+ progenitor cells in CML by increasing mitochondrial oxidative phosphorylation and ROS accumulation. The autophagy genes ATG4a, ATG4b, ATG4c, ATG5 and beclin-1 are overexpressed in CD34+ cells in CML compared with normal cells, suggesting that autophagy participates in the development of CML. Imatinib may induce autophagy or autophagic death in CML, and autophagy is induced in wild-type or BCR-ABL-positive mouse Baf3 cells carrying the T315I mutation. This effect may be related to endoplasmic reticulum stress (ERS), suggesting that imatinib may mediate both apoptosis and autophagic cell death in CML cells. However, as mentioned above, autophagy represents a potential protective mechanism for CML stem cells, allowing them to escape from imatinib-induced apoptosis, as manifested by imatinib resistance. Resveratrol is a natural phytoprotective compound present in grapes and is currently undergoing clinical trials in patients with some metabolic diseases, epithelial tumors, and hematological malignancies, including multiple myeloma and follicular lymphoma. The main mechanism of action is to induce autophagic cell death. Resveratrol induces both apoptosis and autophagy in CML cells and is thought to overcome the chemoresistance of imatinib. Resveratrol-mediated autophagy in CML does not depend on beclin1, but requires AMPK and the activation and upregulation of p62/SQSTM1 transcriptional activity. According to other studies, this polyphenolic compound functions at different levels of the PI3K/AMPK/mTOR axis.

43.4.5 In Chronic Lymphocytic Leukemia (CLL), Deacetylase Inhibitor MGCD0103 Reduces the Level of Autophagy in CLL Cells

Activation of the PI3K/AKT/mTOR pathway and caspases lead to the degradation of autophagy components and participate in MGCD0103-mediated inhibition of autophagy. In addition, MGCD0103 directly regulates the expression of key

autophagy-related genes at the transcriptional level. Research highlights the therapeutic potential of MGCD0103 as an autophagy inhibitor in CLL (El-Khoury et al. 2014).

43.5 Conclusions

Autophagy plays an important role in the biological behaviors of tumors. Numerous studies have shown that autophagy promotes both cell survival and cell death. Autophagy is characterized by an intricate signaling network involving many key signaling molecules and engages in cross-talk with other physiological metabolic processes in cells. To date, numerous studies have not been able to answer the fundamental question about the two functions of autophagy. However, targeted autophagy will undoubtedly remain a promising tool in anti-tumor therapy. Chemotherapy drugs induce apoptosis and/or autophagy, and patients whose tumors are resistant to apoptosis-inducing drugs, such as patients with CML who are resistant to TKIs, may be sensitive to drugs that induce autophagy. The AMPK/mTOR signaling pathway is a potential new target for tumor therapy. Inhibition of the PI3K/mTOR pathway or alterations in AMPK activity may promote autophagy and/or autophagic cell death. Currently, autophagy-regulating drugs have entered clinical research for the treatment of CML, multiple myeloma and B-CLL and other blood tumors. Since a variety of signaling pathways are involved in regulating autophagy, methods to accurately control and regulate the key molecules of the signaling pathways that induce the autophagic death of tumor cells require further exploration. Subsequent research should focus on explaining the role and status of autophagy in cell survival and death, and determining whether autophagy directly causes cell death, survival or secondary changes in cells in specific environments. In addition, targeted autophagy for anti-tumor therapy inevitably affects the physiological metabolism of normal cells. Selective induction of the autophagic death of tumor cells will be a key issue to be addressed in targeted therapy. An investigation of autophagy under hypoxic conditions may be a better starting point. Solid tumors may be hypoxic and deficient in nutrients during angiogenesis or distant metastasis. Therefore, strategies designed to regulate autophagy may selectively remove tumor cells. Another more challenging area is to clarify the roles of autophagy in the different stages of leukemia and to obtain an understanding of which stages of tumor formation require autophagy to protect tumor cells. At the same time, an effective method for evaluating whether autophagy is the cause or result of changes in cellular metabolic processes is needed. If autophagy is only a secondary change, drug resistance occurs due to the activation of autophagy during drug-induced tumor cell apoptosis. Therefore, specific pathways that activate autophagy in different leukemias and other cellular processes associ-

ated with autophagy (such as cell differentiation and apoptosis) must be elucidated by analyzing the interactions to accurately identify targeted treatments and reverse tumor growth. By obtaining a complete understanding of the complex molecular mechanisms that influence the process of autophagy, we will be able to design more precise autophagy-regulating drugs to determine which patients will benefit from autophagy-related therapies (Evangelisti et al. 2015).

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Chapter 44
Autophagy and Lymphoma

Zhong Zheng, Li Wang, Shu Cheng, Yan Wang, and Weili Zhao

Abstract Lymphoma is a hematological malignancy and its incidence is growing. The use of CD20 monoclonal antibody improves the therapeutic efficacy in CD20-positive B-cell lymphoma. Despite remarkable progress in lymphoma treatment over the past decades, chemotherapy resistance and disease relapse become the main obstacles to further improve the prognosis of the patients. Therefore, the development of new treatment methods and drugs is urgently needed to improve the treatment of lymphoma. In tumors, autophagy functions to protect tumor cells from hypoxia, radiotherapy, and apoptosis. The ability to improve the prognosis of patients with lymphoma through the active regulation of autophagy represents a new approach to clinical treatment.

Keywords Autophagy · Lymphoma · AKT/mTOR pathway · AMPK pathway

Lymphoma has become one of the top ten high-incidence tumors worldwide. Targeted therapy has become a hot topic in clinical treatment. By targeting tumor-specific genes, treatment is more precise and the side effects are effectively reduced. The use of CD20 monoclonal antibody to treat lymphoma improves the therapeutic effect and prognosis of patients with CD20-positive B-cell lymphoma, but the treatment effects on patients with CD20-negative lymphoma remains poor. Therefore, the development of new treatment methods and drugs is urgently needed to improve the treatment of lymphoma.

44.1 Autophagy and Lymphoma

Many genes are involved in lymphoma and are closely related to autophagy. The three genes listed below are the most frequently studied genes:

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44.1.1 Beclin1

Beclin1 is a homologue of yeast Atg6, which is located on human chromosome 17q21 and plays an important role in the formation of autophagic vacuoles. Beclin1 deletion leads to a decrease in autophagy and subsequently leads to tumorigenesis. In Beclin1 knockout mice, the incidence of spontaneous breast cancer, lung cancer, liver cancer, and lymphoma increases significantly. In patients with lymphoma, the degree of deletion of beclin1 is significantly associated with lymphoma invasion and prognosis (Willenbacher et al. 2015).

44.1.2 Bcl-2 Family Molecules

Bcl-2 family molecules, such as bcl-2, bcl-xl, bcl-w, and Mcl-1, not only inhibit cell apoptosis but also bind to beclin1 and inhibit the pro-autophagy function of beclin1. The binding of beclin1 to PI3K is inhibited by the BH3 domain of the bcl-2, thereby preventing the initiation of autophagy. Therefore, the mechanism regulating the interaction between beclin1 and bcl-2 is considered a critical step in the initiation of autophagy. The interaction of Bcl-2 and beclin1, the "transformer" of cell homeostasis, regulates cellular autophagy and survival (Tilija Pun and Park 2018).

44.1.3 Tp53

The Tp53 protein is a well-known tumor suppressor protein, and more than half of human malignant tumors have a deletion or mutation in the Tp53 gene. Tp53 is the most effective tumor suppressor gene discovered to date and plays an important role in DNA damage repair, cell cycle regulation, aging, and apoptosis. It is also involved in regulating autophagy signaling pathways, positively or negatively regulates autophagy (Campo et al. 2018). The overexpression of Tp53 or activation of Tp53 by DNA damage inhibits the mTOR signaling pathway and promotes autophagy. Researchers postulate that nuclear Tp53 promotes autophagy by upregulating DNA damage-regulated autophagy modulator (DRAM). In some cases, Tp53 also functions as a negative regulator of autophagy, as autophagy is activated when Tp53 is inactivated. The localization of Tp53 in the cell determines its effect on autophagy. The restoration of Tp53 expression in the Tp53-deficient cytoplasm effectively inhibits Tp53 deletion-induced autophagy; however, the restoration of Tp53 in the nucleus does not effectively inhibit autophagy (Aubrey et al. 2016). Therefore, Tp53 has a variety of regulatory functions in autophagy, but many problems remain unresolved.

44.2 Autophagy Is a Double-Edged Sword for Lymphoma

Autophagy is defined as the response of tumor cells to external stress. Based on accumulating evidence, autophagy promotes and inhibits tumorigenesis and progression.

First, autophagy is a protective mechanism that protects tumor cells from stress induced by low nutrient levels and therapy-induced damage. During tumor progression, tumor cells, particularly cells inside the tumor, activate a self-preservation mechanism to escape death in the absence of oxygen and the presence of limited nutrients due to the reduction in the blood supply. The VEGF-C/NRP-2 pathway is involved in hypoxia-induced autophagy, and the use of VEGF-C or NRP-2 inhibitors to downregulate autophagy promotes tumor cell death and reduces drug resistance. In addition, the autophagic degradation of mitochondria prevents the spread of pro-apoptotic factors, such as cytochromes and apoptosis-inducing factors, thereby preventing apoptosis. Autophagy also helps tumor cells to reduce the accumulation of reactive oxygen species (ROS) caused by radiation or ionizing radiation, clear damaged organelles, and escape death. Tumor cells that are deficient in apoptosis will undergo autophagy under nutrient-deficient conditions. If autophagy is simultaneously inhibited, the cells will undergo necrosis. Therefore, autophagy is a self-survival mode of cells in harsh environments. Inhibition of autophagy increases the cytotoxic effects of drugs (Song et al. 2017). When tamoxifen was used to activate Tp53 in lymphoma cells, the tumor volume in the mouse model showed a transient decrease and then relapsed. Further studies in mice with lymphoma found that when Tp53 is activated, the number of apoptotic cells is substantially increased and a large number of autophagic cells appear at the same time. If the autophagy inhibitor hydroxychloroquine or an ATG5 siRNA is used in combination with the activation of Tp53 to inhibit autophagy, Tp53-induced apoptosis is increased. Similarly, the use of hydroxychloroquine in combination with cyclophosphamide to treat lymphoma also promotes the massive death of lymphoma cells. Therefore, the combination of autophagy inhibitors and apoptosis inducers in the treatment of tumors substantially increases the sensitivity of tumor cells to apoptosis-inducing agents (Lu et al. 2016).

Second, as type II programmed cell death, autophagy also represents a promising treatment method using strategies that promote tumor autophagy. Beclin1 is an important gene involved in autophagy that is expressed in many tumors. The initiation of tumor autophagy inhibits tumor proliferation and induces tumor cells to enter a persistent autophagy state through multiple pathways. Under this condition, autophagy is becoming a new treatment.

44.3 Autophagy and Lymphoma Treatment

44.3.1 AKT/MTOR Pathway Inhibition

AKT/mTOR pathway is an important pathway promoting lymphoma proliferation. This pathway is highly activated in both T-cell and B-cell lymphoma and is associated with lymphoma invasion and resistance. Rapamycin binds to FK506 binding protein and forms a complex that directly inhibits the mTOR pathway, arrests the cell cycle, and inhibits proliferation (Li et al. 2014).

Ono et al. used CD19 antibody-chelated liposomal rapamycin to treat Burkitt lymphoma cell lines in vitro, including SKW6.4, Raji, Namalwa, and two primary Burkitt lymphoma cell lines. The results showed a significant reduction in the numbers of viable cells. The inhibition of proliferation is directly proportional to the concentration of the drug. At the same time, the autophagy marker protein LC3 was detected, and the levels of LC3-II increased significantly as the drug concentration increased. Further application of the pan-Caspase inhibitor Z-VAD-FMK inhibited the apoptotic pathway and antioxidant N-acetyl-L-cysteine (NAC) inhibited the ROS pathway, but these effects were completely inhibited by the autophagy inhibitor 3-MA, indicating that the mechanism by which CD19 chelated liposomal rapamycin inhibits Burkitt lymphoma is not mediated by apoptosis or necrosis, but is mediated by autophagy. Similarly, rapamycin inhibits the growth of a variety of tumor cells, such as glioblastoma, small cell lung cancer, pancreatic cancer, breast cancer, and B-cell lymphoma, and the role of apoptosis is not obvious (Shimobayashi and Hall 2014).

44.3.1.1 Temsirolimus

Temsirolimus is an analogue of rapamycin with good solubility characteristics. Therefore, it has been investigated clinical trials for various tumors and exerts a significant inhibitory effect on proliferation. Since cyclinD1 regulates the downstream target molecules of the AMPK pathway in the autophagy-related pathway and the mantle cell lymphoma is based on cyclinD1, clinical studies related to targeted autophagy have been conducted in subjects with mantle cell lymphoma. Mechanistic studies have shown that temsirolimus inhibits lymphoma proliferation, but the effect is not significant and the tumor progresses further, which may be related to tumor escape from autophagy and compensatory activation of the AKT pathway caused by mTOR inhibition (Atilla et al. 2017). The administration of a combination of drugs further decreases the activity of the AKT/mTOR pathway, inhibits lymphoma cell proliferation, and promotes autophagic death. Thus, methods designed to improve the therapeutic effect have become a main research focus in the treatment of lymphoma.

Histone deacetylase (HDAC) inhibitors are a class of complexes that promote histone acetylation and regulate tumor gene expression by acetylating histones and through epigenetic modifications. These inhibitors block tumor growth and have

attracted increasing attention in clinical research. Valproic acid (VPA) is a clinically used anti-epileptic drug and HDAC inhibitor of short-chain fatty acids. Abnormal HDAC expression is often observed in tumors and is one of the mechanisms by which tumor cells promote self-proliferation and inhibit autophagy. In lymphoma, HDAC inhibitors significantly inhibit the proliferation of B-lymphoma cells, and exert a certain effect on patients with relapsing and refractory disease. When VPA is administered in combination with the mTOR inhibitor temsirolimus, it exerts a synergistic effect on inhibiting the proliferation of Burkitt lymphoma cell lines. Furthermore, the apoptosis markers AnnexinV and Caspase3 were detected. The combination treatment group did not exhibit a significant increase in apoptosis, but increased expression of LC3. Based on these results, the synergistic inhibition of the proliferation of Burkitt lymphoma by the two drugs is not mediated by apoptosis but is mediated through autophagy. The combination of the two drugs inhibited mTOR to a greater extent. The addition of VPA attenuated the feedback AKT activation caused by temsirolimus and inhibited the AKT/mTOR pathway more effectively. The expression of beclin1 in lymphoma cells was significantly increased following treatment with the combination of the two drugs, and a greater number of autophagic vacuoles were observed using electron microscopy. These findings were further verified in vivo in the mouse Burkitt lymphoma model, as VPA combined with mTOR inhibitors effectively inhibited the growth of Burkitt lymphoma. Based on a pathological examination of lymphoma tissue from animal models, (i) the proliferation rate of lymphoma cells (ki67-positive cells) decreased significantly in the two-drug combination group, (ii) the number of apoptotic cells (TUNEL-labeled cells) did not increase, and (iii) the electron microscopy examination showed a significant increase in autophagy. These results further confirmed that the combination of HDAC inhibitors with mTOR inhibitors synergistically inhibits the AKT/mTOR pathway and activates autophagic death, providing new insights into the treatment of lymphoma. In addition to Burkitt lymphoma, similar results were obtained when diffuse large B-cell lymphoma cell lines were treated with VPA combined with temsirolimus (Dong et al. 2013). In mantle cell lymphoma, the administration of another HDAC inhibitor, vorinostat, significantly increases the inhibitory effect of temsirolimus on the proliferation of mantle cell lymphoma and promotes the autophagic death of malignant cells.

44.3.1.2 Everolimus

The rapamycin analogue everolimus is an oral mTOR inhibitor. According to preclinical studies, everolimus increases the chemosensitivity of lymphoma cell lines, such as mantle cell lymphoma and diffuse large B-cell lymphoma. Patients with refractory lymphoma have been included in stage I/II clinical trials. Johnston et al. reported the results from 19 patients with refractory relapsed Hodgkin's lymphoma. These patients received a median number of six treatments; 84% of patients relapsed after autologous transplantation and 47% of patients responded, of which eight reached partial remission and one achieved complete remission with a median progression of

7.2 months. Everolimus displayed therapeutic efficacy in 30% of patients with refractory non-Hodgkin's lymphoma. In addition to lymphoma, everolimus also exerts a certain therapeutic effect on refractory CLL and macroglobulinemia. Similar to rapamycin and temsirolimus, everolimus mainly inhibits mTORC1 and has no effect on mTORC2, leading to the activation of AKT by the mTORC2 pathway and subsequently attenuating the therapeutic effect of everolimus. Preclinical studies of the administration of everolimus combined with chemotherapy to treat mantle cell lymphoma have shown that everolimus significantly increases the sensitivity of mantle cell lymphoma cell lines to chemotherapy drugs such as doxorubicin, vincristine, and targeted drugs, including rituximab, bortezomib, vorinostat, etc. (Merli et al. 2015).

44.3.1.3 Other mTOR Inhibitors

Although the mTOR inhibitor only targets mTORC1, it causes the feedback activation of mTORC2-AKT, which alters the therapeutic effect of the drug. Will the administration of a mTOR inhibitor with mTORC1 and mTORC2 inhibit tumors growth to a greater extent? Torin1 competes with ATP for binding to mTOR and simultaneously inhibits the properties of mTORC1 and mTORC2, showing significant inhibitory effects on tumor growth in preclinical studies. XL-765 and PI-103 are both PI3K-mTOR inhibitors with efficacy against advanced-stage solid tumors and metastatic breast cancer in phase I/II clinical trials, and these compounds inhibit cell proliferation to a greater extent than Rapamycin. CAL-101, a PI3K inhibitor, also exerts superior therapeutic effects compared to rapamycin in phase I clinical studies of refractory lymphoma (Heras-Sandoval et al. 2014).

44.3.2 Activation of the AMPK Pathway

44.3.2.1 Hypoglycemic Drugs

The AMPK pathway is often inhibited in lymphoma. The use of drugs to activate the AMPK pathway inhibits the mTOR signaling pathway and the subsequent proliferation of lymphoma cells. Metformin is an oral hypoglycemic agent that activates the AMPK signaling pathway. According to a large number of epidemiological studies, treatment with metformin reduces the incidence of many tumors, such as breast cancer, prostate cancer, colon cancer, and pancreatic cancer. Preclinical studies have also shown that metformin inhibits the proliferation of primary lymphoma cells and multiple T/B-lymphoma cell lines, such as SU-DHL-4, Namalwa, DB, SU-DHL-5, Daudi, Jurkat, 6-TCEM, H9, and HUT78 cells, among others, but has little effect on the proliferation of normal hematopoietic stem cells. Furthermore, metformin inhibits lymphoma cell proliferation through an AMPK-dependent effect on the mTOR pathway, as knockdown of AMPK expression abolishes the inhibitory effect. The use of mTOR inhibitors usually causes the compensatory activation of AKT, leading to resistance

to mTOR inhibitors. Metformin inhibits the mTOR pathway by activating AMPK and does not cause the compensatory activation of AKT, thereby reducing the resistance to mTOR inhibitors. The combination of metformin and mTOR inhibitors increases the accumulation of LC3 and the formation of autophagosomes in lymphoma cells, rendering lymphoma cells more sensitive to mTOR inhibitors. Metformin increases the sensitivity of lymphoma cells to mTOR inhibitors and chemotherapy drugs, increasing the effect of chemotherapy drugs on lymphoma cells. In addition, patients with hyperglycemia who are treated with the HyperCVAD regimen for highly invasive lymphoma/leukemia, such as lymphoblastic lymphoma, Burkitt lymphoma and ALL, have been investigated in randomized clinical trials of hypoglycemic agents. One group is concurrently treated with HyperCVAD and insulin to lower the blood glucose levels. The other group is treated with traditional oral hypoglycemic agents, such as metformin or thiazolidinedione, along with HyperCVAD. The duration of remission of patients receiving insulin hypoglycemic therapy is significantly shorter, resulting in a poorer prognosis than patients receiving metformin (Vu et al. 2012). Similarly, insulin, particularly insulin glargine, activates the AKT/mTOR pathway in malignant lymphocytes, thereby promoting cell proliferation and resistance to chemotherapy. Metformin and rosiglitazone inhibit the AKT/mTOR pathway and increase the sensitivity of malignant lymphocytes to etoposide, asparaginase, vincristine, and other chemotherapeutic drugs (Pan et al. 2012).

Phenformin is another diterpene hypoglycemic drug that activates the AMPK pathway in an LKB1-dependent manner. Phenylpyrene not only activates AMPK but also inhibits the phosphorylation of AKT. In a murine model, phenformin significantly inhibits the proliferation of a variety of tumors, including lymphoma. The rational use of hypoglycemic agents by patients with lymphoma/leukemia presenting with high blood glucose levels activates the AMPK pathway and increases sensitivity to chemotherapy, providing new ideas and methods for clinical treatment (Rajeshkumar et al. 2017).

44.3.2.2 Natural Products

Berberine is a natural isoquinoline alkaloid and a traditional Chinese medicine ingredient that effectively activates the AMPK pathway, inhibits fat synthesis, and promotes fatty acid oxidation. Berberine effectively promotes AMPK phosphorylation in tumor cells, induces ROS production, and inhibits ERK phosphorylation, ultimately leading to reduced numbers of tumor cells and significantly reduced adhesion and migration.

Curcumin is also a natural product that inhibits tumor cell proliferation by regulating a variety of cellular signaling pathways, such as activating the AMPK pathway and caspase cascade and inhibiting the mTOR and NF-κB pathways. Curcumin activates the AMPK pathway and does not compensate for the activation of the AKT pathway. Instead, it inhibits the AKT pathway and the NF-κB pathway to block the proliferation of lymphoma cells (Zhu and Bu 2017).

44.3.2.3 New AMPK Activators

5-Aminoimidazole-4-carboxamide-riboside (AICAR) is an adenosine analogue that promotes autophagy by activating AMPK in a variety of lymphocytic leukemia cell lines, such as CCRF–CEM, NALM6, REH, and SupB15 cells. Notably, AICAR leads to the compensatory activation of the AKT/mTOR pathway, thereby attenuating the inhibitory effect of AICAR on cell proliferation. Therefore, researchers recommend the administration of AICAR in combination with mTOR inhibitors to minimize malignant lymphocyte proliferation. In addition, the combination of AICAR and methotrexate enhances MTX-induced endoplasmic reticulum stress and increases the accumulation of activated oxygen free radicals, thereby promoting the apoptosis of malignant lymphocytes (Dembitz et al. 2017).

44.4 Conclusions

Autophagy is a highly conserved metabolic process that enables cells to survive in a stressful environment. In tumors, autophagy functions to protect tumor cells from hypoxia, radiotherapy, and apoptosis. Autophagic death caused by multidrug inhibition of the AKT/mTOR pathway or activation of the AMPK pathway has become a trend in cancer therapy research. New AKT-mTOR inhibitors and AMPK activators have also entered clinical trials. The ability to improve the prognosis of patients with lymphoma through the active regulation of autophagy represents a new approach to clinical treatment.

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Chapter 45
Autophagy and Myeloma

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Abstract Multiple myeloma is a hematological malignancy. It is characterized by the abnormal clonal proliferation of malignant plasma cells in the bone marrow and the secretion of a large number of monoclonal immunoglobulins or light chains, causing bone destruction, elevated blood calcium levels, anemia, and renal dysfunction. Autophagy has a dual role in the autophagy of myeloma cells. On the one hand, autophagy eliminates abnormal proteins and organelles in cells, prevents gene damage, and inhibits tumorigenesis. On the other hand, once tumors are formed, tumor cells use autophagy to ensure their survival under nutrient-deficient and hypoxic conditions. Excessive autophagy promotes another form of death in tumor cells, autophagic cell death. Targeted autophagy is becoming another new myeloma treatment strategy.

Keywords Autophagy · Apoptosis · Multiple myeloma

Myeloma is a serious threat to human health and has become the second most common hematological tumor after lymphoma. Although proteasome inhibitors, such as bortezomib, and immunomodulators such as thalidomide and lenalidomide, have substantially improved the disease-free and overall survival rates of patients, they still do not cure the disease. The medical community is searching for other effective treatments for myeloma.

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45.1 Autophagy and Myeloma

45.1.1 The Unfolded Protein Response, Ubiquitin Proteasome Pathway, and Autophagy in Myeloma

Myeloma cells are characterized by a large number of synthetic and secreted monoclonal immunoglobulins and light chains, which are likely to cause the production and accumulation of a large number of unfolded and misfolded proteins that exceeds the ability of the endoplasmic reticulum to fold proteins and subsequently causes endoplasmic reticulum stress (ER stress). Myeloma cells activate the unfolded protein response (UPR), induce the degradation mediated by the proteasomal pathway, and promote autophagy to remove these potentially toxic excess folded and unfolded proteins and to avoid damage caused by excess and persistent endoplasmic reticulum stress. Notably, a complex interlaced connection exists between these pathways for protein clearance. The unfolded protein response (UPR) is achieved by activating three transmembrane proteins on the endoplasmic reticulum. These three transmembrane proteins include glycosyl regulatory enzyme (glycosyl regulatory enzyme corrected as inositol-requiring enzyme 1) (IRE1), PKR-like ER kinase (PERK), and activated transcription factor 6 (ATF6). Usually, the proteins bind to glycomodulatory proteins (GRP78/Bip) and are in an inactive state. When excess unfolded proteins are present in the cell, GRP78 dissociates from the complex and the transmembrane protein is activated. IRE1 comprises a kinase domain and an endogenous ribonuclease region, the latter of which is the insertion site for the transcription factor X-box binding protein 1 (XBP1). XBP1 upregulates the expression of the ER expansion gene and the gene encoding the ER chaperone protein, increases the ability of the endoplasmic reticulum to fold proteins, and activates the AKT-mediated anti-apoptotic signal. On the other hand, the IRE1 kinase region is autophosphorylated, activates the downstream JNK pathway in which Bcl-2 is phosphorylated by JNK and the autophagy-related beclin1 protein binds to PI3K Class III to promote autophagy. PERK is the most important component of the UPR and plays an important role in inhibiting protein translation and reducing protein entry into the endoplasmic reticulum. PERK activates eukaryotic initiation factor 2α (eIF2α) through phosphorylation, inhibits eIF2β, blocks eIF2 activation, and inhibits the translation of most proteins. However, the selective translation of some specific mRNAs, such as ATF4, has been retained. Downstream of ATF4, the pro-apoptotic transcription factor CHOP (CADD153) activates GADD34, which dephosphorylates eIF2α through PP1, a negative feedback regulator. ATF4 also upregulates the autophagy genes LC3B and Atg12, and CHOP upregulates Atg5. In addition, ATF4 increases autophagy by inhibiting the PI3K Class I pathway, in which ATF4 inhibits mTOR through Redd1 and CHOP inhibits AKT through Trb3. ATF6 exists as two subtypes, α and β, but the UPR is mainly directed toward α subtype. Upon ATF6 activation, the protein is transported to the Golgi apparatus and cleaved to expose a nuclear localization signal. In the nucleus, ATF6α regulates the transcription of the ER gene, promotes endoplasmic reticulum expansion, corrects endoplasmic reticulum stress, and allows more

proteins to be folded. ATF6 also increases autophagy through the Akt-independent activation of Rheb and mTOR, thereby maintaining cell survival and linking the UPR to autophagy pathways (Cubillos-Ruiz et al. 2017; Gardner et al. 2013).

The ubiquitination and proteasome pathway is the main pathway for intracellular protein degradation. When the UPR regulates the endoplasmic reticulum to eliminate excess unfolded proteins, protein degradation is induced by the ubiquitin proteasome pathway in tumor cells and the autophagic lysosomal pathway is activated. The great success of proteasome inhibitors (such as bortezomib) in the treatment of myeloma suggests that the ubiquitin proteasome pathway is abnormally activated in myeloma cells. Moreover, the inhibition of the proteasomal degradation of unfolded proteins, which results in the accumulation of toxic proteins in myeloma cells, sustained endoplasmic reticulum stress, and apoptosis. Prolonged endoplasmic reticulum stress induced by proteasome inhibitors induces the development of protective autophagy in tumor cells (Kao et al. 2014). The administration of autophagy-inducing drugs or compounds in combination with autophagy inhibitors has the potential to induce apoptosis, as most of these drugs exert synergistic effects, but exceptions have been noted in treatments employing the proteasome inhibitor bortezomib. The administration of bortezomib in combination with the autophagy inhibitor 3-MA in the early initiation phase produced antagonism and decreased apoptosis. Inhibition of autophagy using siRNAs targeting LC3B and beclin-1 also antagonized the effects of proteasome inhibitors. When bortezomib is combined with bafilomycin A1, a late autophagy inhibitor that inhibits autophagosome and lysosome fusion, it increases apoptosis induced by endoplasmic reticulum stress, and the compounds exert synergistic effects. Moreover, endoplasmic reticulum stress induced by bortezomib alone appears within 8 h, and endoplasmic reticulum stress induced by bafilomycin A1 appears 48 h later. Therefore, if U266 cells are pretreated with bafilomycin A1 before adding bortezomib, apoptosis is induced. Above phenomenon that does not occur with the simultaneous administration of the two drugs. The complex autophagy pathways induced by the application of proteasome inhibitors in tumor cells remains unclear. Importantly, autophagy-inducing agents promote the initiation of autophagy, and when a large number of autophagosomes are formed, a late autophagy inhibitor is added to inhibit autophagosome and lysosomal fusion and degradation of the contents. Degradation of the material will not provide energy for the tumor cells. A large number of autophagy processes burden the cells, increasing cytotoxicity. Proteasome inhibitors increase autophagy in myeloma cells, but they inhibit autophagy in bone marrow mesenchymal stem cells, which facilitates the differentiation of mesenchymal stem cells into osteoblasts and promotes the repair of bone lesions.

45.1.2 Autophagy and Apoptosis in Myeloma

The relationship between autophagy and apoptosis is more complex, as synergistic, antagonistic and interdependent effects have been observed. (i) Autophagy is

essential for apoptosis: When the novel immunosuppressive agent FTY720 is added to myeloma U266 cell line, it causes apoptosis and simultaneously increases the expression of the autophagy marker LC3B-II. Thus, FTY720 induces apoptosis and autophagy simultaneously. Apoptosis decreased after the application of the autophagy inhibitor Baf added to cells, suggesting that autophagy promotes apoptosis. The possible mechanism is that autophagy degrades anti-apoptotic factors, such as Survivin or upstream regulatory factors in lysosomes, which promotes apoptosis. (ii) Autophagy inhibits apoptosis and protects cells: Rubescensine A induces apoptosis and autophagy in myeloma RPMI8226 cells, which is accompanied by an increase in intracellular ROS levels and nuclear levels of the SIRT1 protein. Apoptosis increases after the administration of the autophagy inhibitor 3-MA, suggesting that Rubescensine A-induced autophagy inhibits apoptosis and protects tumor cells. Apoptosis is the main pathway that induces RPMI8226 cell death. (iii) Autophagy and apoptosis are prerequisites: As_2O_3 induces autophagy during the process of apoptosis in RPMI8226 cells. Moreover, in experiments using 3-MA to inhibit autophagy, autophagy was identified as a prerequisite for the caspase-dependent apoptotic pathway. When autophagy is inhibited, apoptosis is also inhibited. Experiments using zVAD-FMK to inhibit apoptosis show that apoptosis is a prerequisite for autophagy, as the inhibition of apoptosis inhibits autophagy (Zhou et al. 2016).

45.1.3 Caspase-10 and Autophagy in Myeloma

Previously, caspase-10 and caspase-8 were thought to only play a role in promoting apoptosis, but new studies have shown that caspase-10 is also a key molecular in autophagy to promote survival or death in myeloma cell lines. Survival is required for this process (Lamy et al. 2013). Usually, autophagy in myeloma cells maintains survival, and caspase-10 prevents the occurrence of autophagic death caused by autophagy. In myeloma cells, caspase-10 and its related protein $cFLIP_L$ are regulated by the transcription factor IRF4. IRF4 is regulated by proto-oncogenes and is expressed at high levels in myeloma cells, which induces the expression of caspase-10 and $cFLIP_L$ and the production of the caspase-10/$cFLIP_L$ heterodimer, thereby activating the functions of Caspase10 as a proteolytic enzyme. Activated caspase-10 cleaves downstream proteins, and one of its most important substrates is BCLAF1. BCLAF1 binds to the anti-apoptotic proteins in the BCL-2 family and promotes apoptosis. BCL-2 inhibits autophagy by binding to beclin1. Under normal circumstances, when caspase-10 is activated, it cleaves BCLAF1 and inhibits the binding of BCLAF1 to BCL-2 to regulate excessive autophagy. Caspase-10 inhibition will theoretically disrupt the balance of autophagy, facilitate autophagic death, and achieve the purpose of treating tumors (Dong et al. 2016).

45.2 Autophagy and Myeloma Treatment

45.2.1 The Role of Autophagy Inhibitors in the Treatment of Myeloma

45.2.1.1 Application of Autophagy Inhibitors

The adriamycin-resistant myeloma cell line RPMI8226/DOX exhibits an increased number of autophagosomes and increased levels of the autophagy marker LC3II, suggesting that autophagy is involved in the resistance of myeloma to doxorubicin. The administration of doxorubicin in combination with the autophagy inhibitor chloroquine or 3-MA effectively induces apoptosis in the RPMI8226/DOX cell line. Similarly, the application of the DNA-damaging drug doxorubicin or melphalan to the myeloma cell lines H929 and RPMI8226 induces caspase-dependent apoptosis. In addition, due to bcl-2 inhibition, beclin-1-mediated protective autophagy is induced. The use of autophagy inhibitors enhances the sensitivity of tumor cells to DNA-damaging drugs.

The PI3K/mTOR/AKT pathway is the most important and active pathway involved in the pathogenesis of multiple myeloma. Drugs targeting this pathway have been the focus of myeloma treatment. At the same time, as mentioned above, mTOR exerts a negative regulatory effect on autophagy, and drugs that inhibit this pathway will increase protective autophagy in tumor cells. In the study by Aronson, PI-103, a dual inhibitor of PI3K and mTOR, effectively inhibited the downstream proteasome pathway, but was accompanied by a compensatory increase in autophagy, which affected apoptosis. When the MM1S and H929 myeloma cell lines were treated with the combination of PI-103 and the autophagy inhibitor bafilomycin A1, autophagy was inhibited and apoptosis was increased.

Sorafenib is a multitarget receptor tyrosine kinase inhibitor that induces the death of the myeloma cell lines LP1 and RPMI8226. The sorafenib-treated myeloma cell line exhibited a decrease in the levels of p62 (a protein degraded by autophagosomes) and an increase in the esterification of LC3, suggesting an increase in autophagy. Combined with the autophagy inhibitors 3-MA and chloroquine, sorafenib effectively induces the death of myeloma cell lines, suggesting that sorafenib-induced autophagy exerts a tumor-protective effect on myeloma because sorafenib inhibits autophagy and increases cell death.

45.2.1.2 Compounds that Inhibit Autophagy

Calpain effectively blocks the protective autophagy induced by bortezomib and enhances the antitumor effect of bortezomib. Calpain inhibits autophagy by inhibiting the fusion of autophagosomes and lysosomes, thus increasing the cytotoxicity of bortezomib.

Macrolide antibiotics, such as clarithromycin, azithromycin, and erythromycin have a similar structure to the autophagy inhibitor bafilomycin A1; thus, these compounds also inhibit autophagy. Bortezomib was applied in combination with clarithromycin or azithromycin to the myeloma cell lines IM-9, U266, and RPMI8226, and increased cytotoxicity was observed. This effect was achieved by the two-way inhibition of the proteasome and autophagy pathway, resulting in increased aggregate formation, the intracellular accumulation of ubiquitinated proteins, and upregulation of the pro-apoptotic transcription factor CHOP following endoplasmic reticulum stress.

45.2.2 Treatments that Promote Autophagy in Myeloma

Glucocorticoids play an important role in the treatment of myeloma. Dexamethasone was an effective treatment for the myeloma cell line lp1. Dexamethasone induced autophagy via a PML/AKT-dependent pathway and induced autophagic death to promote tumor cell death.

The iron chelator, deferasirox, exerts an antitumor effect on myeloma or primary cells by inducing autophagy. Autophagy is achieved by inhibiting mTOR. After myeloma cells were treated with an iron chelator, the level of phosphorylated p70S6 kinase, which is downstream of mTOR, is decreased, indicating that mTOR is inhibited.

A high level of NAD+ turnover is observed in tumor cells, and FK866 depletes NAD+ to cause myeloma cell death by promoting autophagy. The autophagic death program in this myeloma cell line is activated by two mechanisms. First, it inhibits the MAPK pathway, promotes the nuclear localization of the transcription factor EB (TFEB), and upregulates the expression of autophagy-related genes. Direct inhibition of the PI3K/mTORC1 pathway induces autophagy.

45.3 Conclusions

A large number of unfolded proteins and misfolded proteins are present in myeloma cells, which depend on the unfolded protein response to regulate the function of the endoplasmic reticulum in a timely manner. If the regulatory mechanism fails, the proteins are ultimately eliminated by the proteasome and autophagy to reach equilibrium. The great success of proteasome inhibitors in the treatment of myeloma suggests that future treatments should disrupt the balance of protein metabolism in tumor cells. As our understanding of autophagy in the pathogenesis and development of myeloma improves, autophagy regulators will become the new treatment options in the future.

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Part XI
Autophagy in Trauma

Trauma is common in modern society. Central nervous system (CNS) trauma, such as traumatic brain injury (TBI) and spinal cord injury (SCI), is one of the most common causes of long-term disability and death among young adults in the world. They induce an enormous physical, emotional, and economic burden on both the individuals and society. The mechanisms underlying TBI and SCI include initial necrosis of irreversibly damaged cells in the impact area, followed by the delayed secondary cell death in the neighboring penumbral regions. Among posttraumatic secondary biochemical responses, signs of autophagy have been observed. In this part, we update the current state of knowledge and recent advances in the study of autophagy after trauma including TBI, SCI, heart and lung trauma, fracture, and skin injury which we try to clarify how autophagy levels and flux are affected by injury or trauma and how their manipulation may represent potential novel protective targets for trauma treatments.

Chapter 46
The Function and Mechanisms of Autophagy in Traumatic Brain Injury

Chengliang Luo and Luyang Tao

Abstract Traumatic brain injury (TBI) is one of the most common causes of long-term disability and death worldwide. Autophagy is activated and autophagic flux is impaired following TBI. But the controversial roles and underlying mechanisms of autophagy after TBI are not clear. This chapter will update the current state of knowledge in the process of autophagy, the roles of autophagy in TBI as well as some upstream moleculars and pharmacological regulators of autophagy involved in TBI. We also discuss autophagy mechanism-based preclinical pharmacological intervention. These observations make autophagy an attractive therapeutic target for developing new therapeutic strategies to achieve better outcomes for patients suffering from TBI.

Keywords Autophagy · Autophagic flux · Traumatic brain injury · Cell death

46.1 Introduction

Traumatic brain injury (TBI), one of the most common causes of long-term disability and death worldwide, causes cell death and behavioral deficits, imposing a significant burden on society. The pathology of TBI includes the primary injury (which is refractory to treatment) and the secondary injury. The latter is characterized by cellular and biochemical that mainly consists of apoptosis autophagy, oxidative stress, and inflammation (Luo et al. 2011). Brain trauma initiates delayed progressive tissue damage through a cascade of molecular and cellular events leading to neuronal cell death. There have been limited advances in the therapeutic strategies to counter brain injury. Except for conservative management, neuroprotection and neural recovery are still the main therapeutic strategies. Apoptosis has been attributed to programmed neuronal cell death in TBI. Interestingly, recent studies including ours have shown that autophagy is enhanced following TBI (Lai et al. 2008; Luo et al. 2011; Zhang

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et al. 2008). Autophagy inhibition using its inhibitors could attenuate cerebral damage and functional outcomes following TBI (Luo et al. 2011). However, the roles of autophagy in TBI and underlying mechanisms still need to be further addressed in the future.

Autophagy is an evolutionarily conserved pathway that causes degradation of proteins and entire organelles in cells undergoing stress. There are three types of autophagy: macroautophagy (hereafter simply called autophagy), microautophagy, and chaperone-mediated autophagy (Klionsky 2015). Microautophagy involves the direct engulfment of the cargo by the lysosomal membrane. Chaperone-mediated autophagy is characterized by transfer of cytosolic proteins with a KFERQ motif to the lysosome by chaperone proteins, followed by their direct import via the lysosomal-associated membrane protein type 2A (LAMP2A) translocation complex. Macroautophagy, is the most studied type of autophagy and involves the formation around the cargo of a double-membrane vesicle named the autophagosome that subsequently fuses with the lysosome to form autolysosomes (Klionsky 2005). Notably, p62 is regarded as one of the specific substrates that are degraded via the autophagy process. This degradation is mediated by interaction with LC3 (a mammalian homologue of Atg8). LC3 is then recruited to the phagophore/isolation membrane and remains involved in he completed autophagosome (Zheng et al. 2009). To investigate the underlying mechanisms of autophagy, 3-methyladenine (3-MA) and bafliomycin A1 (BFA) are used as selective inhibitors of autophagy. The former is a inhibitor of the Class III PI3K, which can interact with Beclin-1 to participate in the formation of autophagosomes. The latter inhibits vacuolar H^+-ATPase thereby inhibiting autophagy (Luo et al. 2011).

46.2 Autophagic Activation and Autophagic Flux Blockade or Enhancement After TBI

Recent studies have shown that autophagy is increased after TBI (Lai et al. 2008; Zhang et al. 2008). As a marker of autophagy, LC3-II has become a hallmark for measuring autophagy because it is present only within autophagosomes. The increased LC3 immunostaining was observed mainly in neurons at 24 h post-TBI (Liu et al. 2008), and peaked at 6 h in our TBI model (Luo et al. 2011). To evaluate the role of autophagy in TBI-induced cell death and behavior deficits, by using 3-MA and BFA, we found that inhibition of autophagy could reduce cerebral damage and behavior outcome deficits (Luo et al. 2011).

Autophagic cell death has been regarded as one types of programmed cell death. However, whether it causes cell death due to autophagy or not remains controversial. During the conditions of nutritional deficiency, autophagy is used to produce amino acids and energy to maintain cell viability via the degradation of cytoplasmic material. The presence of autophagy in dying cells has been proposed to be a stress response mechanism to prolong cell viability accordingly. Recent studies strongly

support autophagy as a pathway that can induce programmed cell death. Many of the original studies describing autophagy-induced death relied on the observation of autophagy in dying cells and did not examine autophagic flux. Increased expression of autophagy markers such as LC3-II alone is not sufficient to conclude activation of autophagy. Instead, the authors proposed that the autophagosomal formation, maturation, and degradation cycle, known as autophagy flux, may be impaired to create the accumulation of autophagosomes and their marker proteins. The term "autophagic flux" has been used to describe the autophagy as a dynamic process, encompassing the entire process of autophagy holistically. This would include autophagosome formation with subsequent maturation followed by fusion with lysosome, breakdown, and dispensing of toxic proteins and damaged organelles back into the cytosol (Zhang et al. 2013).

Enhanced autophagic flux may be protective after TBI, whereas the inhibition of autophagic flux may contribute to cell death, indicating disruption of autophagy is regarded as one part of the secondary injury mechanism (Sarkar et al. 2014). In autophagic flux studies, while the concept of autophagic cell death is based on observations of increased morphological features (e.g., accumulation of autophagic vesicles) in dying cells. Currently, autophagy-dependent cell death has been described as a novel form. It not only meets the criteria in claim (i.e., independent of apoptosis or necrosis, blocked by autophagy inhibition), but also demonstrates its unique morphological features and a unique ability to be inhibited by pharmacological inhibition of Na^+/K^+-ATPase (Liu et al. 2013). Since SQSTM1/p62 is concentrated within autophagosomes and is normally degraded after lysosome fusion, their accumulation showed the disruption of autophagy flux. Decreased levels of SQSTM1 have been reported to accompany LC3-II accumulation in a mouse models of contusive TBI (weight-drop model) (Luo et al. 2011). Increased autophagy flux was also observed in a mouse focal cerebellar lesion model, confirmed using LC3-II flux assay usually carried out in vitro, LC3-II protein levels during the flux assay, are compared in the presence or absence of a lysosomal inhibitor such as chloroquine (CQ), Bafilomycin A (BFA), or E-64d/pepstatin A (Lipinski et al. 2015). When flux is high, CQ (or any other lysosomal inhibitor) leads to additional accumulation of LC3-II. When flux is blocked, CQ cannot further increase LC3-II levels (Lipinski et al. 2015).

Autophagic flux is important for intracellular "refreshing," and this role in homeostasis is particularly crucial for the health of terminally differentiated cells such as neurons and oligodendrocytes. Increasing studies reported that blocking of autophagic flux in neurons results from lysosome defects or the failure in fusion between autophagosomes and lysosomes, and causes the development of some CNS diseases or injury. The presence of impairment in autophagic flux in the injured cortex after TBI depends on the severity of trauma. Zeng et al. (2008) showed that following moderate and severe TBI, autophagic flux is impaired but not mild TBI, the prognosis of brain injury in a mild injury patient will improve when the initiation of autophagy is enhanced because autophagic flux is normal. When autophagic flux is impaired, as in patients with moderate and severe TBI, strategies that suppress the initiation of autophagy will decrease the accumulation of autophagosomes and induce the fusion of autophagosomes with lysosomes, promoting autophagosome

clearance and subsequently attenuating cerebral damage. Therefore, it is reasonable to treat patients differently according to the severity of trauma. In addition, early after TBI impaired autophagy may play a detrimental role. Treatments that either decrease pathological accumulation of autophagosomes or enhance their degradation may be neuroprotective following TBI. Autophagic clearance is impaired early after TBI due to lysosomal dysfunction, and involves in cell death. Endoplasmic reticulum (ER) stress is also regarded as a potent trigger for autophagy (Lee et al. 2015). The response of ER stress, triggered by various conditions, is a cellular process that disturbs the folding of proteins in the ER (Lee et al. 2015). Moreover, mutations in the PERK phosphorylation site of eIF2a prevents Atg2 upregulation and conversion of LC3 further supports the notion that PERK pathway is a mediator of autophagy. It seems that ER stress-induced autophagy could be neuroprotective based on the observation that the ER stress inhibitor SA could inhibit autophagic activation and exert neuroprotection after ischemic preconditioning.

46.3 Interactions Between Autophagy, Apoptosis, and Necroptosis

A breakthrough has been made in the understanding of the role of Bcl-2 in controlling autophagy via its interaction with Beclin-1 (Sadasivan et al. 2008). Autophagy is activated, and can coexist or occur sequentially with apoptosis. The process of autophagy acts to both increase and decrease apoptosis. Autophagy limits apoptosis through degradation of pro-apoptotic stimuli such as damaged mitochondria or cytotoxic protein aggregates. As a caspase-independent cell death mode, necroptosis exists as an alternative form of programmed cell death when caspase-dependent apoptosis is blocked. Necroptosis is a form of regulated necrosis activated downstream of the tumor necrosis factor receptor 1 (TNFR1), dependent on the activity of the receptor interacting protein kinase 1 (RIPK1) and 3 (RIPK3) and mediated by the mixed lineage pseudo-kinase MLKL. Recent studies demonstrate involvement of necroptosis in neuronal and glial cell death after SCI, and that its inhibition can improve functional recovery in animal models of SCI (Liu et al. 2015a, b). However, the mechanisms leading to necroptosis activation after SCI and its relationship with other cellular pathways remain unclear. Autophagy is a probable downstream consequence of necroptosis rather than a contributing factor to necroptosis and is activated as a cleanup mechanism for cell death. Autophagy inhibitors also suppress Z-VAD-FMK induced cell death, and knockdown of autophagy related genes (e.g., beclin-1 and atg7) were shown to suppress necroptosis in L929 cells, suggesting there is an interaction between necroptosis and autophagy.

As an inhibitor of receptor-interacting protein-1 (RIP-1), necrostatin-1 (NEC-1) was used to potently inhibit necroptotic cell death and became a hot topic of potential therapeutic agential. NEC-1 was expected to reduce TBI-induced disruption of brain tissue and improve functional outcomes (You et al. 2008). Our previous study

found in a mouse TBI model: (1) NEC-1 inhibited TBI-induced Beclin-1 and LC3-II upregulation which maintained p62 at high level; (2) NEC-1 pretreatment also reversed Bcl-2 and caspase-3 expression following, as well as the Beclin-1/Bcl-2 ratio; (3) Both NEC-1 and 3-MA inhibited caspase-3 increase and LC3-II formation, whereas Z-VAD only suppressed caspase-3 level but unregulated LC3-II expression following TBI (Wang et al. 2012). The data demonstrated that multiple cell death mechanisms involved in the development of TBI, but NEC-1 suppressed apoptosis and autophagy simultaneously. The interrelationship among necrosis, apoptosis, and autophagy may can further explain how NEC-1 attenuate TBI-induced cerebral damage and functional outcomes.

There is a complex crosstalk between autophagy and apoptosis. The autophagy function of the Beclin-1 class III PI3K complex is inhibited by Bcl-2. Previous work has shown that certain autophagy inducers (like starvation) disrupt the binding of Beclin-1 to Bcl-2 thereby freeing the pro-autophagic Beclin-1 to stimulate autophagy, by activating signaling pathways (such as JNK1-mediated phosphorylation of Bcl-2) rather than by altering the total protein levels of these two proteins. Another study has indicated that phosphorylation of viral Bcl-2 may not be required for binding to Beclin-1 and autophagy inhibition during starvation. Our previous work reported that both 3-MA and BFA could suppress the ratio of Beclin-1/Bcl-2, and level of active caspase-3 expression following TBI (Luo et al. 2011), demonstrating autophagic inhibition exerts neuroprotection at least partly by suppressing apoptosis.

46.4 The Relationship Between Autophagy and ER Stress

Although ER stress and autophagy are enhanced and involves in neural cell death following TBI, the relationship between them is quite intricate. Most studies suggested that autophagy exerts pro-survival effects following ER stress, but a severe and persistent of stress can cause autophagy-dependent cell death. Prior studies including ours have reported that ER stress-induced cell death was largely associated with upregulation of ATF4 and CHOP expression (Gao et al. 2018). Specifically, ATF4 and CHOP can regulate the autophagy gene (including Beclin-1 and p62/Sqstm1) transcription program in stressed cells. IRE1-JNK signaling pathway also involved autophagy activation via Bcl-2 phosphorylation, enabling the dissociation of Beclin-1 and the activation of the PI3K complex. Another study has reported that ER stress-induced autophagy might contribute to the neuroprotective effect of brain ischemic preconditioning. In summary, the activation of PERK–eIF2α–ATF4–CHOP pathway may be involved in regulating autophagy after TBI in our study (Wang et al. 2019). But the underlying molecular mechanisms and the crosstalk between ER stress and autophagy after TBI need further research.

The molecular crosstalk between ER stress and autophagy represents a cycle that can be pharmacologically targeted to inhibit cell death following acute injuries to CNS, including TBI. ER stress induces the formation of autophagosome through IRE1–JNK signaling pathway. Moreover, mutations in the PERK phosphorylation

site of eIF2a prevents Atg2 upregulation and conversion of LC3 further supports the notion that PERK pathway is a mediator of autophagy. SAL (an ER stress inhibitor) could suppress autophagic activation and provide neuroprotection following ischemic preconditioning. In our previous study, IL-33/ST2L signaling is suppressing autophagy and ER stress (Gao et al. 2018). But the crosstalk between ER stress-induced autophagy and apoptosis following TBU and the underlying mechanisms need to be further addressed.

46.5 The Dual Role of Autophagy in TBI

Although autophagy enhancement after TBI has been found in both animal (Liu et al. 2008; Luo et al. 2011) and human (Clark et al. 2008) models, the role of autophagy in TBI was still controversial. Different studies may draw different or even opposite conclusions. Increased autophagy is observed in multiple and distinct experimental models of brain injury including trauma (Smith et al. 2011). However, it is not clear whether the dual role of autophagy is good or bad after brain injury. It seems that autophagy's role is dependent on the cell's capacity to respond in relation to the cumulative burden of dysfunctional or damaged macromolecules and organelles. If the increase in autophagic capacity is insufficient, augmenting autophagy would likely be beneficial. The increase in autophagic capacity is in excess, and inhibition of autophagy may be beneficial. Therefore, the role of autophagy may be evaluated by whether or not it can meet intracellular demands. TBI causes damage to proteins, lipids, and organelles secondary to activation of proteases and lipases, free radical damage, and a host of other mechanisms.

The protective role of autophagy in TBI was first proposed by Erlich et al. (2007) using rapamycin. They found that rapamycin was neuroprotective following TBI by activation of autophagy. Sarkar et al. (2014) reported that impaired autophagy flux was involved in cell death and apoptosis following TBI. Several drugs exacted neuroprotection by attenuating TBI-induced secondary brain injury via activation of autophagy (Ding et al. 2015).

Support for the detrimental role of autophagy in TBI was initially according to the study conducted by Lai et al. (2008). Our previous study showed inhibition of autophagy by bafilomycin A1 (BafA1) and 3-methyladenine (3-MA), two autophagy inhibitors, attenuated behavioral outcome, reduced cell injury, lesion volume, and apoptosis after TBI, supporting that autophagy was detrimental for TBI (Luo et al. 2011). There were also numerous studies confirming the detrimental role of autophagy in TBI (Tang et al. 2017).

46.6 Autophagy Mechanism-Based Preclinical Pharmacological Intervention After TBI

To promote the neuroprotection and reduce the bad effect after TBI, two methods of autophagy pharmacological intervention were explored. One method observes the change of autophagy using the effective agents for TBI therapy. The other method evaluates the therapeutic outcome using a drug that directly modulates autophagic function after TBI.

46.6.1 Anti-necroptosis Agents

As a newly discovered caspase-independent programmed necrosis pathway, necroptosis can be triggered by activation of death receptor. As a specific inhibitor of RIPK1 and necroptosis, necrostatin-1 (NEC-1) has been a hot topic of therapeutic agent. Rosenbaum et al. (2010) found that NEC-1 could decrease the expression of LC3-II after retinal ischemic. Currently, NEC-1 was reported to change the activities of other types of cell death-associated factors. For instance, (1) NEC-1 suppressed production of reactive oxygen species (ROS), glutamate-induced nuclear translocation of apoptosis inducing factor (AIF), and poly (ADP-ribose) polymerase (PARP) activation; (2) NEC-1 efficiently reduced arachidonic acid (AA)-induced cell death via blocking reactive oxygen species (ROS) production and c-Jun N-terminal kinases (JNKs) activation; and (3) NEC-1 inhibited Z-VAD-FMK-induced phosphorylation of extracellular signal-regulated kinase 1/2(ERK1/2) in a mouse model of Huntington's disease. These findings showed that NEC-1 could alter other cell death modes besides necroptosis, at least in certain conditions. These accessory effects of NEC-1 coincide with the potential role of RIPK-1, a multifunctional protein, which is involved in different pathways of cell death and survival.

Besides reducing the amount of cell injury and tissue damage, NEC-1 has been shown to improve behavior outcomes and attenuate the damage of brain tissue after TBI (You et al. 2008). Moreover, necroptosis was closely associated with autophagy and apoptosis, and thereby, inhibition of necroptosis by NEC-1 may interfere with the pathway of autophagy and apoptosis. Multiple cell death pathways involve in the development of TBI, but NEC-1 suppresses apoptosis and autophagy simultaneously. The interrelationship among necrosis, apoptosis and autophagy may further explain how NEC-1 can reduce tissue damage and functional outcomes following TBI (Wang et al. 2012). These results indicated that autophagy played a detrimental role in TBI.

46.6.2 Membrane-Resealing Agents

The loss in cell membrane integrity, consisting of membrane blebbing and altered permeability, has recently been found to be a major contributor to the development of neuronal damage subsequent to traumatic injury by leading to ionic imbalances and activation of several cellular pathways. Plasmalemma permeability contributes to blood-brain barrier (BBB) disruption, brain edema and cell apoptosis following TBI. Poloxamer 188 (P188) is a nonionic, amphiphilic co-polymer (MW: ~8400), including a central hydrophobic molecule which is flanked on both sides by two hydrophilic chains of polyoxyethylene. Having various clinical applications as a surfactant, P188 has been found to be capable of sealing damaged cell membranes. As an amphiphilic copolymer of polyoxyethylene and polyoxypropylene, P188 is used as a pharmaceutical agent. P188 can seal damaged cell membranes, restore plasma membrane integrity, and have a cytoprotective action in many types of cells. Considering that maintaining plasmalemma integrity is important, P188 may be a potential drug for TBI treatment in clinical applications (Bao et al. 2012).

The effects of plasmalemmal resealing by P188 on neuronal autophagy after TBI was also investigated in our recent study. Plasma membranes could be resealed by P188, and P188 could aggravate autophagy after TBI in vivo (Bao et al. 2016). Moreover, acute membrane damage is a critical precipitating event following TBI. However, the subsequent effects of the mechanical trauma, including mitochondrial and lysosomal membrane permeability, remain elusive. Using cortical and hippocampal neuron cultures, P188 was shown to protect neurons from excitotoxic or oxidative stress-related necrosis and from electroporation by inserting into the membrane and inhibiting membrane peroxidation. Mitochondrial and lysosomal membrane permeability damage was observed in injured neurons, and the mechanism can be investigate with pharmacological treatment. P188's role appears to involve in the relationship between cathepsin B and tBid-mediated mitochondrial initiation of cell death (Luo et al. 2013).

46.6.3 Hydrogen Sulfide

Hydrogen sulfide (H_2S) has been known as a toxic gas characterized by its offensive odor, described as the smell of rotten eggs, and its being an environment pollutant. It had long been assumed that H2S exists in animal tissues at very low concentrations because of its toxicity, although it could be produced endogenously. H_2S is a lipid-soluble, endogenously produced gaseous messenger molecule collectively known as gasotransmitters). In various models of tissue and cellular injury, gasotransmitters have emerged as potent cytoprotective mediators. Endogenous H_2S in brain is produced from L-cysteine by cystathionine-β-synthase (CBS), the pyridoxal 50-phosphate-dependent enzyme.

The changes of H_2S and its possible role in the pathogenesis after TBI were investigated in our recent study. Our data demonstrate that: (1) decrease of endogenous H2S pathway and H_2S level was in parallel with the expression of CBS after TBI; (2) CBS may be implicated in neuronal death and the pathophysiology of brain after TBI; (3) The protective effect and the therapeutic potential of H_2S in the treatment of TBI may be correlated with regulating apoptosis and autophagy (Zhang et al. 2014). In addition, as a novel hydrogen sulfide (H_2S)-synthesizing enzyme, 3-mercaptopyruvate sulfurtransferase (3-MST) may be involved in thiosulfate biosynthesis and cyanide degradation. Currently, considerable attention has been focused on the biochemistry and molecular biology of H_2S-synthesizing enzymes. However, few concerted attempts are done to investigate the changes in the expression of the H_2S-synthesizing enzymes with disease states. 3-MST was found to mainly locate in living neurons, to be implicated in the autophagy of neurons after TBI (Zhang et al. 2014).

46.6.4 Mitochondrial Protective Agents

Mitochondria, the primary energy-generating system in most eukaryotic cells, have been shown to be a crucial participant in TBI pathophysiology, undergoing constant changes in shape and size. Mitochondria are promising therapeutic targets for prevention of cellular dysfunction and death after TBI. Inhibition of the mitochondrial permeability transition pore (mPTP) by cyclosporine A (CsA) is one of the most extensively studied and promising mitochondrial-targeted TBI therapies. Synaptic mitochondria sustain more damage than non-synaptic mitochondria after severe controlled cortical impact injury (CCI), but post-administration of CsA (20 mg/kg) 15 min improves synaptic and non-synaptic respiration, with a significant improvement being observed in the more severely impaired synaptic population (Kulbe et al. 2017). Moreover, mitochondrial morphology is orchestrated by dynamin-related protein 1 (Drp1), dynamin-related GTPases for fission and optic atrophy-1 (OPA1), mitofusions (Mfn1 and Mfn2) for fusion. Drp1 is primarily found in the cytosol, and by interaction with Fis1, Drp1 localizes to discrete spots on mitochondrial surfaces to initiate fission.

Mdivi-1, a mitochondrial division inhibitor, is a highly efficacious small molecule acting as a selective inhibitor of Drp1. Disturbed regulation of mitochondrial dynamics, the balance of mitochondrial fusion and fission, has been implicated in neurodegenerative diseases, such as Parkinson's disease and cerebral ischemia/reperfusion. Nevertheless, the effects of mitochondrial dynamics on TBI have not been exploited. Using mdivi-1, a small molecule inhibitor of Drp1 (a key mitochondrial fission protein), we found that mdivi-1 attenuate TBI-induced cell death and functional outcome by maintaining normal mitochondrial morphology and inhibiting apoptosis activation (Wu et al. 2016).

Our recent study demonstrated that mitochondrial autophagy (mitophagy) specifically occurred at early stage of TBI, with extensively inducing bulk autophagy (Wu et al. 2018). When autophagy flux is enhanced, LC3-II level raises but the level of

p62/SQSTM decreases (Fukui et al. 2012). In parallel, LC3-II accumulation and decreased p62/SQSTM1 level were induced by TBI, but both 3-MA and mdivi-1 reversed these changes in our study (Wu et al. 2018). Autophagy flux was enhanced by TBI, whereas mdivi-1 mitigated the enhanced autophagy flux at 24 h post-TBI. However, mdivi-1 did not suppress TBI-induced ubiquitin increase, although the increase of ubiquitin was reversed by 3-MA treatment. In addition, our study demonstrated mdivi-1-medicated anti-apoptosis following TBI maybe related to autophagic inhibition. The upregulation of LC3II level at 24 h after scratch injury in vitro, suggesting that the insult stimulated autophagy and increased autophagosome abundance. However, preventing autophagosome–lysosome fusion by using chloroquine (CQ) treatment did not result in an additional increase in LC3II level, indicating that the increased LC3II level in the model group may result from the impairment of autophagosome clearance. CQ also abrogated the mdivi-1-induced decrease in LC3II level, demonstrating that mdivi-1's neuroprotection maybe related with inhibiting autophagy dysfunction and increased autophagosome clearance via restoration of lysosomal function (Wu et al. 2018).

46.6.5 Interleukin-33

A variety of mechanisms are involved in cell death, including apoptosis, autophagy, and necrosis. In particular, inflammatory cytokines play a very important role in the development, progression, and prognosis of CNS diseases and injuries.

Interleukin-33 (IL-33) is one of interleukin-1 (IL-1) family cytokines that are produced by many various categories of tissues including the central nervous system (CNS). It was also recognized as an extracellular ligand for ST2, the orphan IL-1 receptor. In recent years, IL-33 is increasingly reported to be involved in the pathogenesis of CNS diseases and injury, such as Alzheimer's disease (AD), multiple sclerosis, chronic pain, intracerebral hemorrhage, and TBI. Interestingly, a recent study demonstrated that levels of IL-33 expression in the brain was elevated from non-detectable levels, reaching a maximum after 72 h in both human TBI samples and mouse TBI model (Wicher et al. 2017). Our recent study showed that IL-33 mediated the inhibition of autophagy and ER stress may be an effective therapeutic strategy for TBI (Gao et al. 2018). The neuroprotective effect of IL-33/ST2L signaling axis on TBI is via inhibiting autophagy, ER stress and apoptosis, and the relationships among them can be quite intricate. The relationship between ER stress and autophagy: ER stress induces the formation of autophagosome through IRE1–JNK signaling pathway.

46.6.6 Salubrina

Salubrinal, a selective phosphatase inhibitor of p-eIF2α (can be phosphorylated by PERK), which was first identified by Boyce and coworkers, suppresses ER stress mediated cell death. By reducing oxidative stress, neuroinflammation and ER-mediated apoptosis, salubrinal could attenuate impulsive-like behavior and improve learning and memory after TBI (Logsdon et al. 2016). But considering the preclinical value, the underling mechanisms of salubirnal need further research in the future.

Although the activation of PERK–eIF2α–ATF4–CHOP pathway was observed after TBI, its involvement in post-injury autophagy was not yet evaluated. Salubrinal significantly decreased the LC3II/LC3I ratio and Beclin-1 level but maintained the P62 level in our TBI model. Besides, double immunostaining showed that salubrinal decreased the number of CHOP+ and LC3+ cells following. These data indicate that salubrinal exerts neuroprotection by suppressing ER stress mediated autophagic cell death after TBI (Wang et al. 2019).

46.6.7 Other Agents: Cathepsin B Inhibitor (CBI), Humanin, and Docosahexaenoic Acid

As one of the major lysosomal cysteine proteases in the intracellular protein catabolism, cathepsin B plays a vital role in apoptotic and necrotic cell death. Enzymatic activity of cathepsin B was significantly increased, and cathepsin B immunoreactivity appeared to be elevated in neurons (Ellis et al. 2005). Specific inhibitors of cathepsin B, such as cystatin A and CBI (a selective cathepsin B inhibitor), can protect cells against ischemic hippocampal neuronal death and excitotoxic striatal cell death. CBI suppressed cell death caused by TBI through the programmed cell necrosis and mitochondria-mediated apoptotic pathways (Luo et al. 2010).

Humanin (HN) is as an endogenous peptide that inhibits Alzheimer disease (AD)-relevant neuronal cell death. HNG, a variant of HN in which the 14th amino acid serine was replaced with glycine, can reduce infarct volume and improve neurological deficits after ischemia/reperfusion injury. Administration of HNG could improve TBI-induced morphological and behavior defects, and downregulate activation of apoptosis and autophagy (Wang et al. 2013).

TBI triggers sustained stimulation of autophagy biogenesis, autophagy flux, and lysosomal functions in the hippocampus. Swift post-injury docosahexaenoic acid (DHA) administration restores hippocampal lysosomal biogenesis and function, demonstrating its therapeutic potential (Yin et al. 2018). In addition, TBI significantly elevated the ATG proteins including lysosomal-associated membrane proteins 1 (Lamp1), Lamp2, cathepsin D (Ctsd), and sequestosome 1 (SQSTM1/p62), which caused decreased cognitive functions together with both gray matter and white matter damages in rats (Yin et al. 2018). DHA treatment inhibited TBI-induced autophagy and reversed the lysosomal biogenesis and function, indicating that autophagy was

detrimental for TBI and inhibition of autophagy exerted neuroprotective effects after TBI (Yin et al. 2018).

46.7 Conclusions

In summary, we have updated the current state of knowledge in post-TBI pathophysiological mechanisms, mainly including programmed cell death mechanisms. We describe the process of autophagy, the role of autophagy in TBI as well as some upstream moleculars and pharmacological regulators of autophagy after TBI. These observations make autophagy an attractive therapeutic target for developing new therapeutic strategies to achieve better outcomes for TBI patients. We also summarize preclinical pharmacological intervention based on autophagy mechanism, including anti-autophagy agents, anti-necroptosis agents, membrane resealing agents, mitochondrial protective agents, anti-inflammatory agents, and others. Moreover, biomarker discovery maybe helpful for us to recognize the earliest symptoms of TBI, and contribute to clinical trials with the patients suffering from TBI.

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Chapter 47
The Function and Mechanisms of Autophagy in Spinal Cord Injury

Chengliang Luo and Luyang Tao

Abstract Spinal cord injury (SCI) is one of the major causes of death and long-term disability in the world. Numerous studies have reported that autophagy plays an important role in SCI. However, the understanding of underlying mechanisms of autophagy after SCI and autophagy mechanism-based preclinical pharmacological intervention needs to be updated. This part provides an overview of current knowledge about the mechanisms of autophagy and autophagy flux as well as its potential molecular mechanisms based on the pharmacological regulation of autophagy. Although autophagic activation and the disruption of autophagy flux exist in SCI, whether autophagy is beneficial and detrimental is still under debate. We also focus on the existing and developing therapeutic options based on the potential molecular mechanisms of autophagy.

Keywords Autophagy · Autophagy flux · Spinal cord injury · Pharmacological agents

47.1 Introduction

Spinal cord injury (SCI) is a highly serious disease of the central nervous system worldwide, which causes the destruction of the motor and/or sensory function leading to temporary or permanent disability. Many of the affected neurons do not die because of direct mechanical damage (the primary injury) but rather show delayed cell death as a result of SCI-induced biochemical changes (the secondary injury). However, there is no effective therapy to treat SCI in clinic up to now. Thus, the disease has brought great challenges to both clinical and scientific researchers.

It is considered as an effective method to treat SCI by attenuating or blocking the secondary injury. Neuronal cell death is an important contributor to SCI-induced neurological deficits. The mechanisms of this death are diverse and include necrosis, classical apoptosis, as well as caspase-independent regulated cell death pathways. In

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2018, the Nomenclature Committee on Cell Death (NCCD) proposed an updated classification of cell death subroutines, consisting of intrinsic apoptosis, extrinsic apoptosis, necrosis, necroptosis, pyroptosis, ferroptosis, parthanatos, entotic cell death, NETotic cell death, lysosome-dependent cell death, autophagy-dependent cell death, immunogenic cell death, cellular senescence, and mitotic catastrophe (Galluzzi et al. 2018). At present, autophagy is increasingly studied in spinal cord injury. Detection of autophagy in spinal cord injury includes the expression of biomarkers and morphological observations. LC3, Beclin-1, and P62 are commonly used for autophagy detection. Morphological methods such as transmission electron microscopy also allow direct observation of the formation of autophagosomes (Kanno et al. 2011). The distribution patterns of autophagy biomarkers are diverse in different cells. In the spinal cord gray matter, autophagic activity mainly concentrated in neurons, while in the white matter autophagosomes mainly accumulated in microglia and oligodendrocytes at 24 h after spinal cord injury.

47.2 Autophagic Activation and Autophagy Flux After SCI

In the autophagic studies of SCI, Kanno and coworkers were the first to report that autophagic proteins Beclin1 and LC3II were overexpressed around the lesion of experimental SCI (Kanno et al. 2009). Notably, the upregulation of Beclin 1 levels was observed starting from 4 h, peaked at 3 d, and lasted for 21 d after SCI (Kanno et al. 2009). Since then, many studies have focused on the mechanisms and effects of autophagy on SCI pathologic processes (Liu et al. 2015).

Impaired autophagic flux is involved in the pathogenesis of numerous neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis. Increased markers of autophagy have also been observed in SCI models. Concomitant accumulation of both LC3-II and SQSTM1 following SCI was described in most reports, indicating more uniform inhibition of autophagy flux after SCI. Diverse injury models were used such as acute contusion SCI in rats and mice, chronic spinal cord compression in mice and rats. The fact that autophagy flux was suppressed even in the mild SCI model may indicate that spinal cord neurons may be more sensitive to autophagy flux perturbations on injury than cortical or hippocampal neurons examined in most TBI models. Moreover, in different types of SCI, the progress of autophagic flux is varied. Autophagy flux is blocked in severe contusion injury, but increased in hemisection injury. In compression injury, autophagic flux is inhibited after severe compression (15 g for 1 min), and enhanced after relatively moderate compression (30 g for 1 min) (Zhou et al. 2017).

The underlying mechanisms by which enhanced autophagy flux contributes to neuroprotection after SCI have yet to be investigated. Trauma could result in generation of damaged cellular components such as lysosomes, mitochondria, and peroxisomes, which is a source of oxidative stress. Reactive oxygen species (ROS) have

been shown to regulate autophagy, depending upon the context and levels. The positive effects of ROS on autophagy flux can be exerted directly by redox-sensitive mediators such as ATG4, or indirectly by upstream regulatory pathways such as the adenosine 5'-monophosphate-activated protein kinase (AMPK). Excessive ROS and reactive nitrogen species (RNS) can suppress autophagy. ER stress is also a potent inducer of autophagy. Liu et al. (2015) reported that autophagosome accumulation after SCI is not due to enhanced initiation of autophagy, but rather due to the inhibition of autophagy flux. This likely reflects the disruption of lysosomal function following SCI, indicating that autophagy is disrupted after SCI and may exacerbate ER stress and neuronal cell death.

It has been previously demonstrated that activation of necroptosis contributes to neuronal and glial cell death after SCI (Liu et al. 2015) and that treatment with RIPK1 kinase inhibitor, necrostatin 1 (Nec-1), can improve cell survival and functional outcomes after injury (Liu et al. 2015). However, the mechanisms leading to activation of necroptosis after SCI and its relationship to other cellular pathways are not clear. A recent report indicated lysosomal dysfunction after SCI may contribute to both inhibition of autophagy and sensitize cells to necroptosis by promoting RIPK1 and RIPK3 accumulation (Liu et al. 2018).

47.3 Crosstalk Between Autophagy and Apoptosis Following SCI

It is well known that apoptosis plays a vital role in SCI-induced axon disruption and cell death. Therapeutic options targeting anti-apoptosis mechanism provide better neurological outcomes in experimental SCI. Numerous literature report that a close biochemical relationship between apoptosis and autophagy exists. Many common signal transduction pathways such as p53 protein, Bcl-2-homology-3-only (BH3-only) proteins, Ser/Thr kinases, and oncogenes influence both autophagy and apoptosis. These processes demonstrate the ability to cross-regulate each other between autophagy and apoptosis through inhibition. Such inhibitory cross talk was found in autophagic studies of SCI, but stimulation of autophagy could significantly downregulate apoptosis and enhance functional recovery (Zhou et al. 2017).

Mainly two methods of autophagy pharmacological intervention in experimental SCI were explored. To promote the neuroprotection and reduce the detrimental effect, one method for experimental SCI therapy evaluates the change of autophagy by using the treatment of an effective drug. The other method evaluates the therapeutic outcome after experimental SCI using a drug that directly modulates autophagic function.

47.4 The Dual Role of Autophagy in SCI

The investigation of changes in autophagy activity after treatment of therapeutic agent believed to result in beneficial outcomes in experimental SCI which can provide indirect evidence for autophagy function. It was resgarded that increase of autophagy after treatment of these therapeutic agents supports the hypothesis that autophagy exerts a protection, whereas the decrease of autophagy supports the contrary. Both 3-MA and Akt inhibitor IV treatment could block autophagic activation and Akt phosphorylation and counteract the antiapoptotic effects of rapamycin and exacerbated neuronal loss (Li et al. 2019), indicating that the neuroprotective effect of rapamycin on rats with spinal cord injury can be achieved by activating autophagy and the Akt signaling pathway. Similarly, rapamycin was observed to be efficient in increasing the levels of LC3 and Beclin1, inhibiting apoptosis and improving functional recovery following SCI. Disruption of autophagic flux with p62 could be enhanced by lysosomal dysfunction after SCI. The facilitation of lysosomal biogenesis by Netrin-1 could improve functional recovery after SCI by increasing TFEB expression via the AMPK/m-TOR signal pathway (Bai et al. 2017). Boosting autophagy stabilized microtubules by degrading a microtubule destabilizing protein, superior cervical ganglia protein 10 (SCG10), in cultured CNS neurons and promoted axon growth. Additionally, treatment with Tat-beclin1 (a specific autophagy-inducing peptide) promoted axon regeneration, attenuated axon retraction, and improved locomotor functional recovery after SCI. This study reveals a critical role of autophagy in stabilizing neuronal microtubules and a beneficial role of autophagy in axon regeneration following SCI.

47.5 Autophagy Mechanism-Based Preclinical Pharmacological Intervention After SCI

Currently, autophagy-related agents have not been applied in the clinical realm. Nevertheless, autophagy is still a potential therapeutic target. Recently, Rong and coworkers used NSC-derived small extracellular vesicles (NSC-sEV) to treat experimental SCI and found they had the potential to reduce neuronal apoptosis, inhibit neuroinflammation, and promote functional recovery at an early stage by promoting autophagy (Rong et al. 2019). With future development of specific strategies for autophagy intervention in different SCI types, the research in this field will be able to improve patient functional recovery after SCI (Zhou et al. 2017).

In contusion injury, the therapeutic agents of atorvastatin and simvastatin, metformin, exendin-4, and vitamins C and E (antioxidants) worked through the mechanisms of apoptosis inhibition, combined with upregulation of autophagy after SCI. Resveratrol, which is synthesized by various medicinal plants in response to injurious substances, has therapeutic effects on neurodegenerative disease, TBI, cerebral

ischemia, and SCI (Liu et al. 2018). Moreover, the neuroprotective effects of resveratrol in SCI are partially through autophagy stimulation and indicated that resveratrol is a promising drug for SCI therapy. Bsperoxovanadium had been reported to provide a neuroprotective effect on SCI by enhancing autophagy via activation of ERK1/2 signaling (Tang et al. 2014). However, methylprednisolone (a synthetic glucocorticoid hormone), valproic acid, and systemic bisperoxovanadium (a small-molecule protein tyrosine phosphatase (PTP) inhibitor) also have effects of downregulating the level of autophagy in the treatment of contusion injury (Seo et al. 2015).

Overall, either inhibition or enhancement of autophagy was reported even when the same SCI model was used after treatment of therapeutic agents. These mixed results cause an ambiguous definition of autophagy's role after SCI, but validating overall effects of autophagy via administering therapeutic agents and analyzing results has several limitations. Therapeutic agents, often, do not directly target the autophagy pathways, and may instead act on a different pathway which may result in a feedback regulation of autophagy. Two main approaches are used for the experimental inhibition of autophagy: interfering with the formation of autophagosomes and blocking lysosomal degradation. Therapeutics that act as agonists or inhibitors in the autophagy pathways would be much more suitable to validate the overall effect of autophagy following SCI. In hemisection injury, 3-MA injection 4 h after SCI at the dose of 2.5 mg/kg led to the declined BBB testing score and increase of apoptosis (Tang et al. 2014). Rapamycin, as a commonly used inducer of autophagy, promoted axonal regeneration toward the lesion, preserved neuronal survival, and improved the restoration of hind limb function after SCI. Similar effects were observed with other autophagy-activating interventions, such as the post-administration of a splicing isoform of human vascular endothelial growth factor A (VEGFA165) (Wang et al. 2015), retinoic acid (which also limited the disruption of the blood–spinal cord barrier) (Zhou et al. 2015), and an anti-atherosclerotic drug simvastatin. Notably, the neuroprotection afforded by VEGFA165 and retinoic acid was abolished by the concomitant administration of 3–MA (Wang et al. 2015) and chloroquine (Zhou et al. 2015), respectively, reinforcing the mechanistic link with autophagy in this setting. However, in many studies discussed above, autophagy was modulated with pharmacological agents that exhibit a low degree of specificity (such as 3-MA and lysosomal inhibitors) (Klionsky et al. 2016). It was difficult to determine whether the protective role of 3-MA was due to its effects on inhibiting autophagy or other pathways. The development of specific agents that regulated autophagy may help to clearly elucidate the role of autophagy in TBI.

47.6 Conclusion

In summary, this part provides an overview of current knowledge about the mechanisms of autophagy and its role in SCI as well as its potential molecular mechanisms based on the pharmacological regulation of autophagy. We summarize the functions of autophagy in SCI and some pharmacological regulators of autophagy involved in

SCI. It is an urgent need for a novel wave of investigations that attempt to dissect the actual impact of autophagy on SCI with rigorous tools. Such tools include flux assays that measure actual autophagic proficiency (as opposed to the accumulation of autophagic markers), pharmacological agents with improved specificity, and genetic strategies that target at least two distinct components of the autophagic machinery in an inducible manner. These investigations or considerations make autophagy an attractive therapeutic target for developing new therapeutic strategies to achieve better outcomes for patients suffering from SCI.

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Chapter 48
The Function and Mechanisms of Autophagy in Trauma of Other Parts of the Body

Chengliang Luo and Luyang Tao

Abstract Trauma is common in modern society. Besides TBI and SCI, trauma can lead to severe cardiopulmonary injury and even to death. Fracture and skin injury are also very likely to occur in our daily life. Limited studies have reported the levels of autophagy after heart and trauma, fracture, and skin injury. In this chapter, we update the current state of knowledge and recent advances in the study of autophagy after trauma including heart and lung trauma, fracture, and skin injury which we try to clarify how autophagy levels are affected by injury or trauma and how their manipulation may represent potential novel protective targets for treatments.

Keywords Autophagy · Heart trauma · Lung trauma · Fracture · Skin injury

48.1 Autophagy in Trauma of Heart and Lung

Trauma can lead to severe cardiopulmonary injury and even to death. With the development of medical technology, the direct mortality rate caused by trauma has been greatly reduced. However, some patients with systemic mechanical trauma have heart events such as myocardial infarction in the days or weeks after discharge. Systemic mechanical trauma can not only cause direct cardiac injury, but also cause delayed cardiac injury. Studies have shown that activation of AMPK pathway by D492 can inhibit mTOR promoting autophagy activation and protect damaged cardiomyocytes (Din et al. 2012). Another in vitro study shows that systemic non-fatal mechanical trauma can lead to a decrease in autophagy level of myocardial tissue, which may be one of the causes of post-traumatic myocardial mitochondrial dysfunction and consequently abnormal cardiac function.

In addition, lung injury is very likely to occur after complex trauma. Animal experimental studies showed that the levels of LC3 and beclin-1 in lung tissue increased 6 h after traumatic brain injury combined with fracture, peaked at 48 h, and maintained a high expression level 5 days after injury. Electron microscopic examination showed

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that the number of autophagic bodies in lung tissue increased (Zhang et al. 2015). By activating autophagy, the expression of ZO-1 and occludin can be upregulated to a certain extent to improve the alveolar epithelial barrier function, thus playing a protective role in acute lung injury.

48.2 Autophagy and Fracture

Fracture healing is a complex and orderly process involving a variety of biological mechanisms. The factors affecting fracture healing include blood supply at the fracture end, the number of osteocytes, the activity of immune cells, and the effect of cytokines (Nelson et al. 2003; Crockett et al. 2011). It was found that autophagy marker LC3 (light-chain 3) was marked by immunofluorescence, and the autophagy level of osteoblasts was significantly higher than that of osteoblasts (Zahm et al. 2011). In hypoxia and nutrient deficiency, osteocytes maintain life activity by activating autophagy. At the fracture end, the survival of autophagic osteocytes is also critical (Manolagas and Parfitt 2010). By establishing a rat fracture model, the researchers found that autophagy marker LC3-II increased gradually 24 h after operation, indicating that autophagy was activated in the process of fracture repair. Rapamycin could inhibit apoptosis by promoting autophagy, angiogenesis, and fracture healing (Yin et al. 2019).

48.3 Autophagy and Skin Injury

The process of skin wound healing includes tissue and cell regeneration and reconstruction. After skin injury, the inflammatory stage begins with cytokines and growth factors, such as transforming growth factor (TGF) β 1/2/3, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) 1/2/4/7/10, epidermal growth factor (EGF), and vascular endothelial growth factor (vascular endothelial growth factor), which are attracted by macrophages and lymphoid tissues and are used to clear the degenerated group. Subsequently, the second stage of proliferation is characterized by granulation and angiogenesis to fill the gap. At this stage, the migration, proliferation, and collagen III synthesis of fibroblasts are regulated by many factors. In the third stage, type III collagen is replaced by type I collagen. Fibroblasts differentiate into myofibroblasts, which play a role in wound contraction and scar maturation. Studies have shown that LC3 positive cells in fibroblasts increased during skin healing, indicating that the autophagic lysosome system participates in the functional changes of fibroblasts during wound healing, but its mechanism and specific role need further research (Asai et al. 2018).

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Part XII
The Progress of Drug Discovery and Therapeutics Targeting Autophagy

In recent years, great progress has been made in relevant research. It has been demonstrated that, in addition to the typical routes for the delivery of autophagic substrates to lysosomes, a novel autophagy strategy that does not depend on core autophagy regulatory proteins had been observed in etoposide-exposed cells. Autophagy has long been recognized as a process of nonselective degradation of large amounts of protein. However, recent studies have shown that autophagy can be selective and this process is closely related to ubiquitin protein and p62 (Zheng and Wang 2010). Studies by Okamoto suggest that Atg32 served as a bipartite platform is able to recruit Atg8 and Atg11 to the mitochondrial surface and to form an initiator complex crucial for mitophagy (Kondo-Okamoto et al. 2012). Their findings may help in screening for selective targets so as to improve the ability to clean up the dispensable or dangerous cytoplasmic material. A majority of drugs developed for targeting autophagy are currently concentrated in cancers, which is the most threatening and challenging disease worldwide. The major obstacles in cancer treatment are the emergency of drug-resistance. Specifically, due to genetic variation or deletion, cells are unable to perform apoptosis properly, resulting in the production of resistance against apoptosis inducer. Therefore, targeting autophagy provides an alternative strategy to develop drugs, which works better for the treatment of cancers. In fact, tamoxifen, rapamycin, and caspase inhibitors have been approved for the treatment of malignant tumors and these drugs have the capabilities of simulation and inhibition autophagy (Levy et al. 2017). In order to give a better understanding of the history of autophagy-related drug development, we briefly outline the relevant research progress in recent years, see Table XII.1.

In recent years, with the deepening of the molecular mechanism of a variety of diseases, the research finds that the pathogenesis of different diseases is quite different and the biomolecules that determine the development process of various diseases show biological specificity. Their relationships are very complicated or even slightly mysterious. Researchers try to exhaust the biological structure of each biomolecule and describe the role of each molecule, but whether it is a macromolecule or a small molecule, it is often stretched from the individual level to study each biomolecule involved in the diseases. It is of convincing necessity to systematically conduct

Table XII.1 A brief history of drug development related to autophagy in recent years

Time	Issue	Reference
1996	Bursch et al. first reported that tamoxifen could treat breast cancer by inducing autophagy.	Bursch et al. (1996)
1997	Blommaart reported that the antiproteolytic effect of wortmannin and LY294002 was accompanied by inhibition of autophagic sequestration via inhibition of C3 PI3K/AKT/mTOR pathway.	Blommaart et al. (1997)
1998	National Cancer Institute (NCI) reported that Tamoxifen decreases the incidence of invasive and noninvasive breast cancer.	Fisher et al. (1998)
1999	Liang found that the autophagy-promoting activity of beclin 1 in MCF7 cells is associated with inhibition of MCF7 cellular proliferation.	Liang et al. (1999)
2000	Si et al. found that matrine has the capability of the formation of autophagosomes.	Si et al. (2000)
2003	Chau et al. found that endostatin predominantly induced autophagic cell death in EAhy926 human endothelial cells.	Chau et al. (2003)
	Kanazawa et al. found that temozolomide could induce reversible autophagy in malignant glioma cell line U3732MG.	Kanazawa et al. (2003)
2004	Gozuacik reported that rapamycin can inhibit the activity of mTOR, thereby inducing autophagy in malignant glioma cells.	Kanazawa et al. (2003); Opipari et al. (2004)
	Peng et al reported that vincristine can induce autophagic apoptosis of HepG2 cells, indicating that drugs induce apoptosis while inducing autophagy.	Peng et al. (2004)
	Ravikumar et al. found that rapamycin or trehalose might enhance the degradation of autophagy, thus autophagy inhibitor might have therapeutic effects on protein deposition disease models such as Parkinson's syndrome.	Ravikumar et al. (2004)
	Cao et al. reported that selenium promoted cell death by endoplasmic reticulum (ER) stress-induced autophagy.	Fakih et al. (2005)
2005	Vignot demonstrated that rapamycin and its derivatives (CC I2779, RAD001, and AP23576) could inhibit mTOR function and cell proliferation in a variety of malignancies.	Vignot et al. (2005)
	Hoyer-Hansen M showed evidence that vitamin D analog EB1089 simulated dramatic lysosomal changes and Beclin 1-mediated autophagic cell death.	Hoyer-Hansen et al. (2005)
	Takeuchi H reported that rapamycin induces autophagy in cancer cells to play a therapeutic role in cancer.	Takeuchi et al. (2005)
	Kanzawa reported that arsenic trioxide induced autophagic death of malignant glioma cells by upregulating the mitochondrial cell death protein BNIP3.	Kanzawa et al. (2005)

(continued)

Table XII.1 (continued)

Time	Issue	Reference
2006	Qin et al. reported that crotoxin simulated autophagic activity and delayed neurotoxin-induced apoptosis.	Yan et al. (2006)
2007	Chang et al. found that concanavalin A (ConA) can induce autophagy in hepatocellular carcinoma cells, and ConA was found to have a therapeutic effect in a mouse model of liver cancer.	Chang et al. (2007)
	Researchers demonstrated that seven FDA-approved drugs could induce autophagy and promote long-lived protein degradation by an image-based high-throughput screen.	Zhang et al. (2007)
	Li et al. confirmed that oridonin could induce autophagy in human squamous cell carcinoma A431 cells. Cui et al. found that oridonin could induce autophagy and inhibit apoptosis in human cervical cancer HeLa cells.	Li et al. (2007); Cui et al. (2007)
	Ertmer et al. found that imatinib-induced autophagy in chronic myeloid leukemia, leading to cell growth arrest and cancer cell death.	Ertmer et al. (2007)
2008	Yang et al. utilized cisplatin to induce the formation of autophagosomes in tubular epithelial cells. Periyasamy et al. found autophagic vesicles and autophagosomes in the rat kidney after intervention with cisplatin. These results lay the foundation for the development of autophagy in cisplatin in kidney disease.	Yang et al. (2008); Periyasamy-Thandavan et al. (2008)
	Cheng et al. found that tumor necrosis factor (TNF-α) can induce autophagic death in mouse fibrosarcoma cell line L929.	Cheng et al. (2008)
2009	Eisenberg et al. found that spermidine can inhibit cell necrosis by modifying and inducing autophagy to prolong the lifespan of animals.	Eisenberg et al. (2009)
	Liu et al. found that *polygonatum cyrtonema* lectin could induce autophagic death in human melanoma A375 cells.	Liu et al. (2009)
	Morselli found that the supply of exogenous spermidine could induce autophagy to prolong the lifespan of yeast, nematodes, and fruit flies. Resveratrol could induce autophagy in the nematode to extend lifespan and keep human cells in good condition under metabolic stress.	Morselli et al. (2009)
2010	Hidvegi et al. found that carbamazepine reduced the liver load of α1-antitrypsin Z and verified the extensive clinical safety of carbamazepine. It also provided a basis for the use of autophagy enhancers as therapeutic agents.	Hidvegi et al. (2010)
2011	Sun et al. found that caspase-independent death was observed in breast cancer MCF-7 cells after treatment with epirubicin. Epirubicin promoted autophagy in human breast cancer MCF-7 cells and protected MCF-7 from epirubicin-induced apoptosis.	Sun et al. (2011)

(continued)

Table XII.1 (continued)

Time	Issue	Reference
2012	Congdon et al. found that thiodiphenylamine methylene blue could induce autophagy and reduce the expression level of tau protein or misfold fold accumulation through related in vitro and in vivo. Thereby it can be used as a drug to treat or ameliorate neurodegenerative diseases such as Alzheimer's disease (AD) and certain forms of Parkinson's disease (PD).	Congdon et al. (2012)
2013	Yu et al. found that Nilotinib induced autophagy in liver cancer cells by activating AMPK. Activation of AMPK was caused by phosphorylation of AMPK, which was regulated by PP2A, and Nilotinib also reduces the activity of PP2A.	Yu et al. (2013)
2014	Zanotto-Filho et al. found that temozolomide and curcumin have synergistic effects in inhibiting malignant glial tumors. They found that ERK1/2 is required for these drugs to induce autophagy in cells.	Zanotto-Filho et al. (2015)
2015	Egan et al. discovered a highly selective UCL1 kinase inhibitor (SBI-0206965) that can inhibit UCL1-regulated phosphorylation and enhance the efficacy of mTOR inhibitors by killing cancer cells via modulating autophagy. Petherick et al. screened two UCL1 inhibitors that could block autophagy by preventing autophagosome maturation.	Egan et al. (2015); Petherick et al. (2015)
2016	DeBosch et al. found that trehalose could induce the occurrence of AMPK-dependent autophagy and effectively prevented the occurrence of fatty liver.	DeBosch et al. (2016)
2017	Levy et al. confirmed that chloroquine inhibits autophagy and improves drug resistance in patients with brain cancer.	Levy et al. (2017)

research from multiple proteins and multiple targets by using systematic biology approaches. Promising opportunities will be attained for the treatment of major human diseases through the network to systematically understand the pathogenesis and treatment mechanism of major human diseases. The systematic biology research on apoptosis has been conducted in depth and relevant progress has been made. Most systemic biology studies on apoptosis have identified some key proteins by network analysis, such as p53 and tumor necrosis factor receptor (TNFR), which can be used as targets for drug development. Although the current systematic biology research on autophagy is still in its infancy, we believe that there will be an increase in systematic biology studies on autophagy. It is expected that proteins that play an important role in autophagy form a basis for further drug screening.

Chapter 49
The Prospects of Therapeutic Potential and Drug Development Targeting Autophagy in Cancer

Jinku Bao, Bo Liu, and Chuanfang Wu

Abstract Autophagy is a self-protection mechanism of cells. Cells can degrade damaged organelles and macromolecules in this way to guarantee the growth and development of cells. In recent years, more and more researches have found that autophagy also plays a certain role in the occurrence and development of tumors. The dual role of autophagy in the development of tumors includes inhibiting the development of tumors; meanwhile, under the condition of insufficient nutrition, autophagy degrades organelles to reduce oxidative stress and provide nutrition and energy for tumor cells so as to protect tumor cells. The regulation of autophagy depends on the development of the tumor, and the corresponding autophagy inducers or inhibitors are constantly emerging, which provides a new direction for tumor treatment.

Keywords Autophagy · Targeted therapy · Drug development

Autophagy is the process by which cells use lysosomes to degrade their damaged organelles and macromolecules. It is an important regulatory mechanism for cell growth, maturation, differentiation, and death, and is also associated with a variety of human diseases including tumors. Some studies have shown that this self-degrading effect and death program can inhibit tumor development. In addition, other studies have shown that autophagy is a self-protection mechanism that protects tumor cells from damage caused by low nutrition, ionizing radiation, and chemotherapy. When tumor cells require insufficient energy and nutrients (such as advanced tumors), autophagy can degrade mitochondria and organelles through lysosomes, alleviate oxidative stress, and provide nutrients and energy for tumor cell growth. Thus, in the

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advanced stage of the tumor, autophagy may be beneficial for tumor cells to grow in a low-vascularized environment. It can be seen that autophagy may play multiple roles due to different stages of tumor development, tissue type, cell differentiation status, surrounding environment, specific genetic characteristics as well as signaling pathways. Further understanding of the regulation mechanism of autophagy will lead to a deeper understanding of the relationship between autophagy and tumor. Due to the dual effects of autophagy in tumors, the induction of autophagy does not necessarily lead to the death of tumor cells which helps to achieve therapeutic effects. Therefore, before the application of autophagy inducers or inhibitors to the clinic, a large amount of in-depth research is required to achieve the purpose of preventing and treating tumors by regulating autophagy.

49.1 Drug Development for Autophagy Signaling Pathways

The molecular regulation mechanism of autophagy plays an important role in tumorigenesis, such as phosphatidylinositol type I and type III (PI3K) pathways, endoplasmic reticulum stress response (ERS), and apoptotic pathways. When tumor cells have developed resistance to apoptotic cell death, anti-tumor by autophagy regulated by the proteins involved in these processes can provide a potential new targeting strategy for the design and development of anti-tumor drugs. Current researches have shown that the anti-tumor effect of many drugs is related to autophagy (Duffy et al. 2015). Certain drugs can activate autophagy directly or indirectly to cause cell death or release some apoptosis-inducing factors through autophagy and lysosomal enzyme activation to induce apoptosis or even necrosis. The response of some tumor cells to anticancer drugs may also be manifested by increased autophagy activity or even autophagic cell death. Therefore, improving autophagy activity of tumor cells is an effective new anti-tumor therapy.

49.1.1 Type I PI3K/Akt/mTOR Pathway

The PI3K/Akt/mTOR signaling pathway has important physiological functions and is involved in the regulation of various biological processes, which include cell cycle, cell survival, protein synthesis, cell proliferation, metabolism, and angiogenesis. Studies have shown that the PI3K (phosphatidylinositol 3-kinase) signaling pathway is upregulated in many types of cancer cells, which is of importance to the induction of autophagy. PI3K-related signaling pathways are involved in cell growth and differentiation, apoptosis, cytoskeletal formation, and cell membrane transport. Both type I and type III PI3Ks have been shown to play a role in autophagy (Kondo and Kondo 2006). Phosphatase and tensin homolog (PTEN) are a PIP3-phosphatase encoded by tumor suppressor gene, which has gene mutation or deletion in a variety

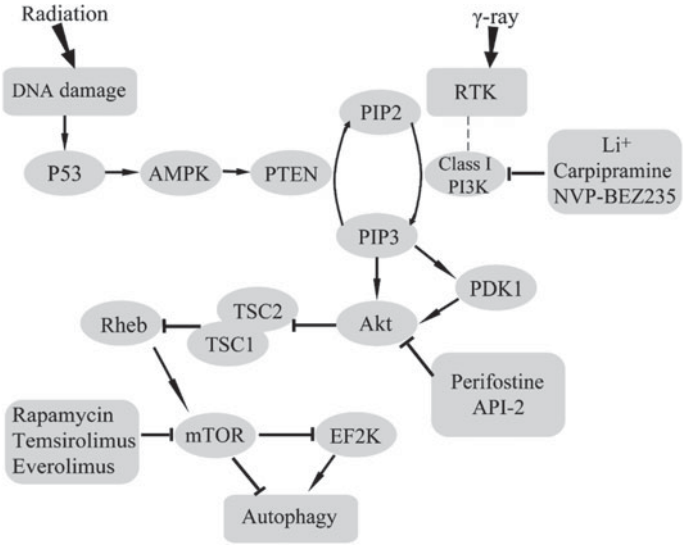

Fig. 49.1 Regulation of type I PI3K-related pathways in autophagy

of tumors. In contrast to the function of PI3K, it can downregulate the Akt/PKB cascade by converting PIP3 to PI-4,5-P2 through dephosphorylation (Fig. 49.1). PTEN reduces Akt activation and blocks all downstream signaling events regulated by Akt.

Currently, the inhibitors targeting the PI3K/AKT/mTOR signaling pathway include broad-spectrum PI3K inhibitors (four subtypes acting on class I PI3K), subtype-specific PI3K inhibitors, PI3K/mTOR dual-targeting inhibitors, and mTOR inhibitors. The first-generation PI3K signaling pathway inhibitors include Wortmannin and LY294002, both of which have IC50 values of 1 nM and 1.4 μM for PI3K in vitro. However, these compounds showed poor pharmacokinetic characteristics and significant toxicity, which limited their potential in clinical therapy. At present, the broad-spectrum PI3K inhibitors in different stages of clinical study include Copanlisib (BAY80-6946), Buparlisib (NVP-BKM120), Pictilisib (GDC-0941), Pilaralisib (XL-147), Sonolisib (PX-866), and Taselisib (GDC-0032) (Janku 2017).

The mTOR/PI3K dual-targeting inhibitor competes with PI3Ks and mTOR1/2 for binding to ATP, which inhibits the PI3K/AKT/mTOR pathway more strongly than inhibition of a single site, thus these drugs can also produce higher levels of toxicity. Currently, PI3K-mTOR dual-targeting inhibitors in different stages of clinical research include Dactolisib (NVP-BEZ235), Apitolisib (GDC-0980), NVP-BGT226, Voxtalisib (XL765), and Gedatolisib (PKI-587) (Liu et al. 2013).

Mammalian target of rapamycin (mTOR) is a downstream effector of Akt/PKB and plays a crucial role in cell proliferation, growth, and survival. Inhibition of mTOR induces autophagy. The first potent inhibitor of mTOR, Rapamycin (RAPA), promotes autophagy and inhibits the growth of malignant glioma cells. The first

approved anti-tumor drugs targeting the PI3K/mTOR signaling pathway were the rapamycin analogs temsirolimus (CCI-779) and everolimus (RAD001), both of which are mTORC1 allosteric inhibitors. The former approved indications for advanced renal cell carcinoma while the latter approved indications include advanced cancer, advanced renal cell carcinoma, advanced pancreatic neuroendocrine tumors, and various cancers.

Rapamycin and its improved analogs temsirolimus, everolimus, and ridaforolimus (AP23573, also known as deforolimus) are used as major mTOR inhibitors in cancer therapy and showed significant chemotherapy inhibition in a variety of models. In previous studies, combined with radiotherapy, mTOR inhibitors significantly reduced the density of hemangiomas in murine models and sensitized vascular endothelium. In addition, studies have shown that everolimus for phase III clinical treatment of advanced renal cell carcinoma can also increase the radiosensitivity of MDA-MB-231 and MCF-7 breast cancer cells (Liu et al. 2012). Meanwhile, everolimus can also increase the radiosensitivity of prostate cancer cells, especially the prostate cancer cell line PC-3 cells that are deficient in the tumor suppressor gene PTEN (Cao et al. 2006). In a variety of human malignancies, PTEN gene deletions or mutations (resulting in decreased autophagy activity) are often present and everolimus is applied to radiation therapy to enhance the efficacy of cancer. In addition, ridaforolimus has also been utilized in the second phase of clinical treatment of advanced blood cancer. These findings suggest a new autophagy target that uses mTOR inhibitors to improve therapeutic efficacy.

In summary, the inhibition of mTOR by rapamycin or its derivatives provides a powerful therapy for various malignant tumors. G protein-coupled receptor (GPCR) antagonists, type I PI3Ks inhibitors (NVP-BEZ235, lithium salt, carbamazepine), Akt inhibitors, Akt/PKB signaling pathway inhibitor-2, and mTOR inhibitors have an induction effect on autophagy and provide a variety of drug targets for tumor therapy. Further study on the role of autophagy in the cytotoxic effects of these compounds will facilitate the treatment of tumors and may develop new combination therapies to achieve anti-tumor effects by specifically inducing autophagy.

49.1.2 Type III PI3K Pathway

Phosphorylation of PI by type III PI3K produces PI3P. Compared with type I PI3K, the combination of type III PI3K and Beclin-1 can help in regulating the formation of autophagosomes and promoting autophagy (Fig. 49.2). Beclin-1 is a membrane-intrinsic protein of the PI3P pathway, in which knockout inhibits autophagy and makes cells sensitive to nutrient-deficient cell death. Related studies have shown that the Beclin-1 gene is absent in 40–75% of breast, ovarian, and prostate cancers (Cao et al. 2006). In addition, increased expression of Beclin-1 in MCF-7 breast cancer cells can induce autophagy, reduce cell proliferation, and inhibit tumor formation in nude mice (Gong et al. 2013). However, loss of heterozygosity of Beclin-1 in mice increases spontaneous malignancy, promotes cell proliferation, and reduces

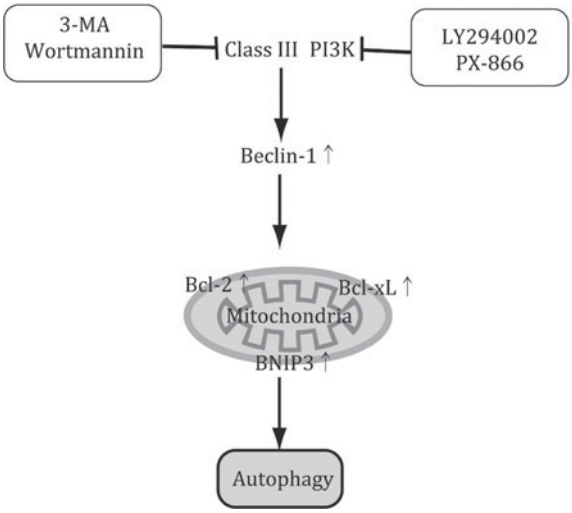

Fig. 49.2 Regulation of autophagy by a type III PI3K-related pathway

autophagy. The potent inhibitors of PI3Ks, 3-Methyladenine (3-MA), Wortmannin, LY294002, and PX-866 reduce the incidence of autophagy while the overexpression of type III PI3Ks connectors and the increase of PI (3) P induced autophagy. These findings have confirmed the importance of Beclin-1 and PTEN in inducing autophagy which has become a potential target for further cancer therapy.

49.1.3 Endoplasmic Reticulum Stress (ERS) Inducer

The ERS response is activated under various stress conditions related to tumor cells, such as hypoxia, hypoglycemia, changes of calcium homeostasis, and accumulation of misfolded proteins. Many cellular stress conditions lead to the accumulation of unfolded or misfolded proteins in the endoplasmic reticulum, which is a potential death threat to cells. To avoid this, unfolded protein response (UPR) is activated by inducing expression of molecular chaperones by inositol requiring-α (IRE1α) and activating transcription factors (ATFs); PKR-like endoplasmic reticulum kinase (PERK) phosphorylates eukaryotic initiation factor 2 (eIF2) to reduce translation and the degradation of misfolded proteins which is increased by the ER-associated degradation (ERAD) system (Sano and Reed 2013). The ERAD pathway upregulates the expression of genes involved in protein degradation. Proteasomes that degrade proteins include proteins related to tumor cell proliferation and apoptosis. Therefore, the proteasome can also be used as a new target for drug discovery (Fig. 49.3).

The ERS response can be induced by different mechanisms and many different drugs have different effects on these pathways. Currently, representative

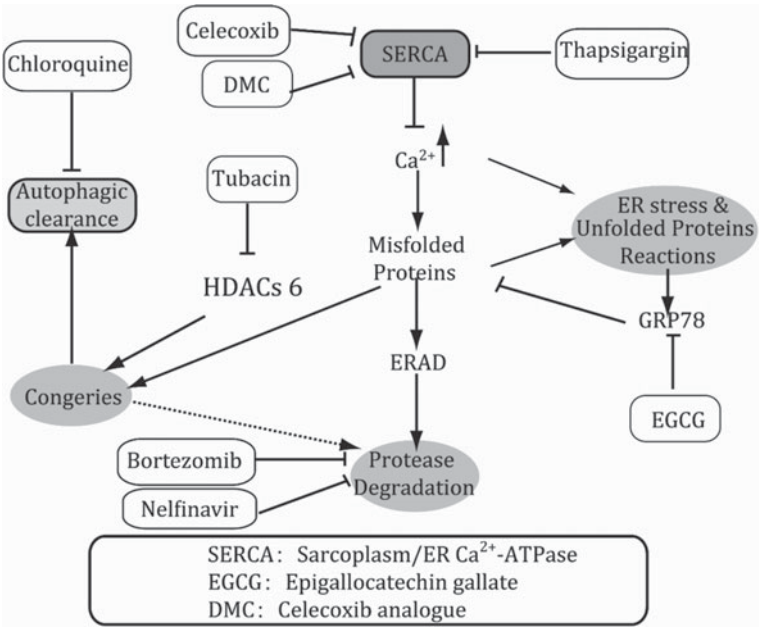

Fig. 49.3 Mechanism of action of ERS and related proteins and targets of drug action

endoplasmic reticulum related drugs include proteasome inhibitors (bortezomib, atazanavir, nelfinavir), SERCA inhibitors (toxic carotenoids, celecoxib, celecoxib analog: DMC), histone deacetylase (HDAC) inhibitors (e.g., tubacin), and other inhibitors (chloroquine, epigallocatechin gallate) (Healy et al. 2009). Therefore, inhibition of autophagy may enhance the ERS response and make tumor cells sensitive to chemotherapy. However, the current results of specific inhibition of autophagy are still controversial, which may be related to the type of inhibitor used and the specific step of inhibiting autophagy. This may be related to the type of inhibitor or the inhibitory step of autophagy.

Since autophagy is involved in the clearance of protein aggregates, inhibition of the clearance process may cause misfolding or unwanted protein aggregation which causes further ERS reactions. Therefore, inhibition of autophagy may enhance the ERS response and make tumor cells sensitive to chemotherapy. However, the current results of specific inhibition of autophagy are still controversial, which may be related to the type of inhibitor used and the specific step of inhibiting autophagy. When the misfolded protein of the entire ER system is overloaded, cells usually produce apoptosis-induced cell death. However, ERS can also induce autophagic cell death (Fig. 49.4). It has been shown that the PERK/ERS pathway can be used as a potential anticancer target or as a cell sensitizer to develop new anticancer drugs.

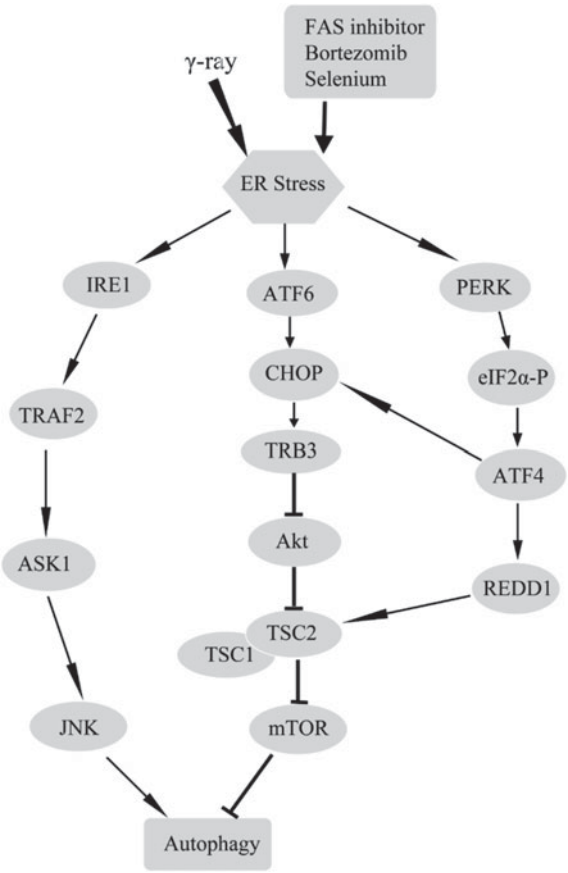

Fig. 49.4 Regulation of autophagy by endoplasmic reticulum stress pathway

49.1.4 Other Autophagy Inducer and Related Signaling Pathways

49.1.4.1 The BNIP3 or Beclin-1 Pathway Acts as Therapeutic Targets for Anti-tumor Drugs

BNIP3 is a member of the BH3-only subfamily of the Bcl-2 family and is involved in the regulation of apoptosis. It is expressed on the mitochondrial membrane and induces cell death by opening mitochondrial permeability transport pores and increasing the production of reactive oxygen species. BNIP3-mediated cell death in breast cancer MCF-7 cells and cervical cancer HeLa cells is independent of apoptotic protease activating factor (Apaf-1), caspase activation, cytochrome c release, and nuclear transfer of apoptosis-inducing factors. In addition, ceramide-mediated autophagic cell death in malignant glioma cells is also mediated by BNIP3. Overexpression of BNIP3 in tumor cells causes autophagic cell death without any other triggering

mechanisms. Moreover, BNIP3 is also involved in autophagy of arsenic-induced glioma cells (Park et al. 2013). This demonstrates the possibility of using BNIP3 as a target for tumor therapy.

The development of new anticancer drugs that target specific signaling proteins of autophagy, combined with drugs currently used and drugs that target key autophagy molecules, can achieve rather effective therapeutic effects. For example, arsenic trioxide has been shown to have potential efficacy in the treatment of a wide range of hematological malignancies such as multiple myeloma, lymphoma, leukemia, neuroblastoma, and malignant glioma (Bonati et al. 2006). The mechanism of arsenic trioxide action encompasses multiple signals, one of which involves activation of the caspase-mediated apoptotic pathway. In malignant glioma cells, arsenic-induced autophagy involves upregulation of BNIP3 (Fig. 49.2). Upregulation of Beclin-1 also occurs in leukemia cell lines treated with arsenic. This suggests that there is a potential pathway through which the efficacy of arsenic can be improved.

49.1.4.2 Death-Associated Protein Kinase (DAPK)

DAPK is a cytoskeleton-associated calmodulin-regulated serine/threonine protein kinase. This enzyme was originally found in interferon gamma-induced apoptosis in HeLa cells. Recent studies have shown that DAPK and DAPK-associated protein kinase (DRP)-1 have autophagic effects in breast cancer MCF-7 cells and cervical cancer HeLa cells (Gozuacik and Kimchi 2006). The expression of these kinases causes vacuolation of the membrane and a wide range of autophagyand this effect is independent of the activity of caspase. Conversely, inhibition of DAPKs reduces the occurrence of autophagy.

In addition, DAPK functions well in inhibiting multiple tumors and inhibiting metastasis. DAPK expression is reduced or eliminated in many human cancers, which can be linked to tumor recurrence and metastasis. DAPK activates a p53-mediated apoptotic pathway that inhibits oncogenic transformation (Fig. 49.5). p53 also regulates autophagy for sure. DAPK also inhibits tumor cell metastasis and infiltration by interaction with integrins and integrin-cdc42 pathway inhibition. From this point of view, the tumor-suppressive effect of DAPKs does not depend solely on apoptosis and may also present multiple ways to combat drug resistance in tumor therapy.

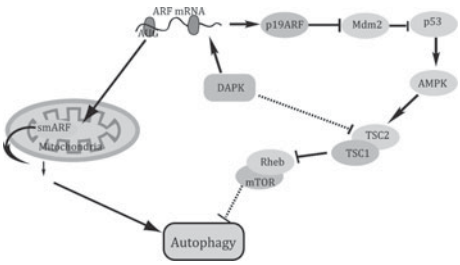

Fig. 49.5 Regulation of autophagy by DAPK-associated pathway

Some traditional drugs, such as thapsigargin, tunicamycin, and brefeldin-A, can induce ERS, but they also have a certain degree of toxicity. Take bortezomib for example. It inhibits the activity of PERK and prevents the degradation of proteasome, resulting in sustained protein synthesis and aggregation of ubiquitinated linked proteins, respectively. These drugs, combined with other strategies (such as radiation therapy), cancan increaseincrease tumor cytotoxicity and avoid the toxicity of surrounding normal tissues at the same time. Selenium may have a selective role in inducing ER stress-mediated apoptosis in tumor cells.

49.1.4.3 Extracellular Signal-Regulated Kinase

Extracellular signal-regulated kinase (ERK) is a member of mitogen-activated protein kinases (MAPKs) and is a serine/threonine protein kinase. Kang et al. showed that ERK transcriptional activity increased the phosphorylated Bcl-2 protein level, and then phosphorylated Bcl-2 dissociated from Beclin-1 to promote autophagy. Ugland et al. developed a novel autophagy pathway associated with Cyclin E, in which cAMP signaling increases ERK activity and upregulates Cyclin E, which in turn promotes aggregation of Beclin-1 around the nucleus, allowing cells to occur. This indicates that ERK plays an important role in regulating the autophagy of cells.

49.1.4.4 Apoptosis Inhibitors that Activate Autophagy as Anti-tumor Drugs

Studies have shown that treated with etoposide (an inhibitor of topoisomerase II) and ionizing radiation, the death rate of mouse embryonic fibroblasts (MEFs) cell which loss of proapoptotic genes Bak and Bax will increase. In addition, when Bak and Bax are deleted, radiation sensitization is enhanced by the increase of autophagy, while autophagy inhibitor 3-MA can induce radiation sensitization. Furthermore, caspase 3/7-deficient MEFs mainly express an increase in radiation sensitization by promoting autophagy and endoplasmic reticulum stress can be activated by radiation. This suggests that the absence of an apoptotic pathway (a marker of cancer development) may lead to autophagic cell death, which may bring about a new cancer treatment strategy.

(1) Bak/Bax inhibitors: Trace element zinc inhibits apoptosis and has also been shown to inhibit Bak/Bax (a gated caspase-mediated cell death). In the past few years, extensive studies have been conducted in radiation-induced Bak/Bax-mediated apoptosis which accounts for less than 20% of cell death. Recent studies have found that the intrinsic apoptotic molecule, Bak/Bax, increases radiation sensitization by inducing autophagy. Moreover, when the Bak/Bax proapoptotic protein is deleted, the radiation sensitization of the cells can be repressed by the 3-MA or Atg5-Atg12 complex and the siRNA that promotes autophagy Beclin-1 specificity. In addition, proapoptotic etoposide can also

induce autophagy in Bak/Bax-deficient MEFs cells. Studies have shown that inhibition of Bak/Bax by zinc is mainly through the induction of autophagy to enhance the induction of cell death by radiation. These studies have also indicated that in radiation-treated cancer cells, Bak/Bax inhibitors can also be used as anti-tumor drugs to induce autophagic cell death in tumor cells.

(2) Caspase inhibitor: z-VAD is a broad-spectrum inhibitor of apoptosis that prevents apoptotic cell death. Recently, evidence embodies that z-VAD also inhibits kinase receptor-interacting protein 1 (RIP1), which indicates that inhibition of RIP1 and caspase is essential for inducing cell death by autophagy. Deletion of z-VAD or caspase-8 can be promoted and deletion of RIP1 can inhibit autophagic cell death. After cell exposure to z-VAD, radiation-induced cell death can be effectively transferred from the apoptotic pathway, replacing it with an autophagic pathway. z-VAD leads to increased radiation sensitization of breast and lung cancer cells, which is associated with increased autophagy signals in breast and lung cancer cell lines. When z-VAD is combined with RAD001, the above cell lines all presented a downward trend in overall cell viability. When used alone, the inhibition of apoptosis by z-VAD and the promotion of autophagy by RAD001 lead to an increase in the radiation sensitization of the cells, which may have an additive but not synergistic effect on cell death. Although the potential cytotoxicity of z-VAD limits the clinical utility of z-VAD, it can also provide evidence for the abovementioned view that caspase inhibitors are used to induce autophagy to fight tumors. Recently, new developments in small-molecule non-polypeptide caspase inhibitors, such as the irreversible broad-spectrum caspase inhibitor IDN-6556, have begun Phase II clinical trials in liver disease. Some studies are now conducting pre-clinical studies by using new caspase inhibitors combined with DNA damaging agents and ERS inducers (such as radiotherapy) in an effort to further evaluate the efficacy and safety of this new method.

49.2 Problems and Prospects for the Development of New Drugs for Autophagy in Cancer Therapy

It requires more clinical verification to answer the question that whether autophagy should be activated or inhibited in cancer treatment. Although autophagy-specific drugs are currently scarce, some compounds that have been shown to affect autophagy may be used for the treatment of cancer (Shi et al. 2013) (Table 49.1).

49.2.1 Autophagy Inhibitors in Cancer Therapy

Many cancer-associated mutations downregulate autophagy, making it easier to target autophagy in cancer cells than in normal cells. A certain degree of autophagy is

Table 49.1 Selected autophagy inducers and inhibitors for cancer treatment

Compound	Target	Inducer/inhibitor	Increase/decrease	Cancer type
HDAC inhibitors (e.g., butyrate and suberoylanilide hydroxamic acid)	Histone deacetylase	Inducer	Increase	Chronic myelogenous leukemia (CML)
Tamoxifen	Estrogen receptor	Inducer	Increase	Breast cancer
EB1089	Vitamin D receptor	Inducer	Increase	In clinical trials against numerous cancers
Angiogenesis inhibitors (Kringle5, ADH-1, AG013736, and anti-VEGF antibody)	Angiogenesis activators	Inducer	Increase	In clinical trials against breast, prostate, brain, pancreas, lung, stomach, ovary and cervix carcinomas
Tyrosine kinase inhibitor (Imatinib)	Several tyrosine kinases	Inducer	Increase	CML
Resveratrol	Multiple targets (e.g., estrogen receptor and mitochondria)	Inducer	Increase	Ovarian cancer
Alkylating agent (temozolomide)	DNA	Inducer	Increase	Primary or recurrent high-grade gliomas
Arsenic trioxide		Inducer	Increase	Acute promyelocytic leukemia and multiple myeloma
Akt-inhibitor	Akt	Inducer/regulation	Increase	
HIV protease inhibitor (ritonavir, saquinavir, nelfinavir)	Protease	Inducer/regulation	Increase	Clinical trial stage I

(continued)

Table 49.1 (continued)

Compound	Target	Inducer/inhibitor	Increase/decrease	Cancer type
mTOR inhibitors (Rapamycin, CCI-779, RAD-OO1, AP23573)	mTORC1	Regulation	Increase	In clinical trials against numerous cancers
Chloroquine	Lysosomotropic drug	Fusion/degradation	Decrease	Antimalarial drugs, developed as sensitizers for radiation therapy and chemotherapy before clinical trials
Monensin	Lysosome PH	Fusion	Decrease	Malignant glioma
Pepsin inhibitor	Lysosomal protease	Fusion	Decrease	Cervical cancer
3-MA	PIK3	Fusion	Decrease	Breast cancer, prostate cancer, colon cancer, malignant glioma, and cervical cancer
Bafilomycin A1	ATPase	Fusion	Decrease	Breast cancer, prostate cancer, colon cancer, malignant glioma, and cervical cancer
Omeprazole	Proton pump	Fusion/degradation	Decrease	Antiacid treatment for digestive diseases
BMS 1, 2, 3 and 4	RabGGT	Fusion	Decrease	
Microtubule interference (vincristine, paclitaxel)	Tubulin	Fusion	Decrease	Numerous tumor types

necessary for tumor growth, so the application of autophagy inhibitors may also have a certain anticancer effect. Synergistic effects may occur when autophagy inhibitors act in combination with other metabolic inhibitors. In order to alleviate the high energy consumption of tumor cells and the low level of oxidative phosphorylation, cancer cells increase glycolysis and activate autophagy. Therefore, an angiogenesis inhibitor or glucose uptake inhibitor in combination with an autophagic blocker might be exploited as a potential anticancer strategy. In addition, since autophagy has important functions in metabolism, it can also eliminate damaged and potentially dangerous organelles. Therefore, the combined application of drugs damaging organelles and autophagy inhibitors may be an effective method for inducing tumor cell death. For example, siramesine (σ-2 receptor antagonist, currently being used as a preclinically developed anticancer drug) damages lysosomes and increases tumor cell death while combined with autophagy inhibitors. In tumor cells, carotene and tunicamycin induce endoplasmic reticulum stress reaction, which causes tumor cell death, while tumor cell death increases when autophagy is inhibited. In addition, blocking autophagy in breast cancer cells also makes them susceptible to camptothecin-induced mitochondrial damage and cell death.

At present, there are very few autophagy inhibitors that have been tested in clinical context. The clinical efficacy of these inhibitors combined with other anticancer drugs needs to be supported by more animal tumor model tests. Inhibition of lysosomal function (and thus autophagy) in a mouse cancer model exhibits additive or synergistic effects in the following combined therapies: proton pump inhibitor omeprazole and cisplatin, chloroquine (currently used as an antimalarial drug), p53 activation or alkylating agents, as well as vincristine and siramesine. When using autophagy inhibitors, it is important to note that they may also act as promoters of tumor development. However, if the tumor-promoting effect of autophagy inhibitors relies on necrotic cell lysis and inflammatory responses, then the combined application of immunosuppressive drugs may prevent this adverse effect.

49.2.2 Autophagy Inducer in Cancer Therapy

Currently, many chemotherapeutic agents (alkylating agents, actinomycin D, and arsenic trioxide), hormone therapy (tamoxifen and vitamin D analogs), natural compounds (resveratrol), cytokine (interferon), gene therapy (p53 and p27 Kip1 genes), and radiation and photodynamic therapy have been shown to trigger autophagic cell death in a variety of tumor cells in vitro, but the anti-tumor effect of autophagy in vivo still needs to be further validated. When apoptosis is missing, autophagy can serve as the primary cell death program. Although autophagy is likely to play a role in promoting survival in normal cell homeostasis, it can also emit a death signal in tumor cells. In order to effectively exert the anticancer effect, the drug that specifically induces autophagy should also be well tolerated. In addition, autophagic cell death can also solve the problem of tumor cell tolerance to apoptosis.

However, drugs that induce autophagy may inhibit tumor cell death caused by inadequate nutrition or other anticancer therapies. For example, rapamycin-induced autophagy inhibits radiation-induced apoptosis, whereas Beclin-1 deficiency promotes siramesine-induced non-apoptotic lysosomal cell death pathways. In addition, the combination of different methods of autophagy induction may increase the sensitivity of tumor cells to autophagic cell death. For example, the vitamin D analog EB1089 can enhance the response of breast cancer cells to radiation. Therefore, the combination therapy involving drugs that induce autophagy needs to be carefully designed. Frequent mutations in genes that regulate autophagy pathways may lead to another therapeutic problem, namely, reduced sensitivity to autophagic cell death. Restoring the levels of Beclin-1 in human breast cancer cells (Beclin-1 gene heterozygote) increases EB1089-induced autophagic cell death. Therefore, adding a drug that upregulates Beclin-1 or other Atg proteins (such as active oxygen generators) may help solve this problem.

49.2.3 Natural Compounds that Induce Autophagy in Tumor

In recent years, some natural compounds that can induce autophagic death in tumor cells have been discovered, such as some small molecules like oridonin, or proteins like Polygonatum cyrtonema lectin (PCL) and Concanavalin A (Con A), etc. Oridonin is an active ingredient extracted from traditional Chinese herbal medicine and has been shown to have significant activity in inducing apoptosis and autophagy. The experiment confirmed that oridonin can induce autophagy in human squamous cell carcinoma A431 cells, and Ras can achieve this autophagy by downregulating the phosphorylation of Akt (a downstream molecule of PI3K). More interestingly, this autophagy does not show typical autophagic features but apoptotic features, such as decrease in mitochondrial membrane potential and upregulation of Bax/Bcl-2 protein ratio, all of which have been confirmed to be involved in this process of autophagy. In addition, it has been shown that oridonin can induce autophagy in HeLa cells. In this process, typical autophagic features such as the transformation from LC3I to LC3II and the increased expression of Beclin1 protein are observed. Decreased expression of Ras and increased expression of P38 and JNK are involved in the regulation of this autophagy.

Some experiments have shown that oridonin can induce apoptosis and autophagy simultaneously in cancer cells, and autophagy can serve as a protective mechanism to protect cancer cells from apoptotic cell death. For example, in L929 cells, oridonin-induced autophagy protects cells from apoptotic cell death, a process that is primarily achieved by upregulating the survival pathway of p38-NF-κB. In addition, in the process of apoptosis and autophagy induced by oridonin, autophagy can play a synergistic role with apoptosis, thus playing a role in promoting cell death. As demonstrated in human breast cancer MCF-7 cells, oridonin can induce apoptosis and autophagy in cancer cells through the MAPK family protein-mediated pathway. Besides, in the human fibrosarcoma HT1080 cells pretreated with oridonin, NF-κB

ultimately promotes apoptosis and autophagic death by activating the p53 pathway. After calpain protease was added to the murine fibrosarcoma L929 cells pretreated with Oridonin, it was observed that apoptosis was inhibited and autophagy was elevated.

All of these results are making oridonin an attractive leading compound in anti-tumor research. More pre-clinical and clinical studies are required for turning oridonin into a new type of effective drug for cancer treatment.

Polygonatum cyrtonema lectin (PCL) has been shown to induce apoptosis and autophagy in human melanoma cell A375. During autophagy, typical features of autophagy, such as the transformation from LC3I to LC3II, and Beclin-1 expression, were observed. Mitochondria are involved in PCL-induced autophagy, autophagy and apoptosis play a synergistic role to promote PCL-induced cancer cell death. In the PCL-induced autophagy through the ROS-p38-p53-mediated pathway. PCL has also been found to induce apoptosis and autophagy in mouse fibrosarcoma cell L929 by inhibiting the Ras-Raf and PI3K-Akt pathways (Fig. 49.6).

ConA has been shown to induce autophagy in hepatoma cell HepG2. Mitochondria is involved in this autophagy process. ConA enters cells by endocytosis and preferentially binds to mitochondria, causing a decrease in mitochondrial membrane potential, which may be related to ConA-induced autophagy. During ConA-induced autophagy of HepG2 cells, AKT expression was downregulated, and typical autophagic features such as formation of LC3II, formation of BNIP3 protein, and appearance of

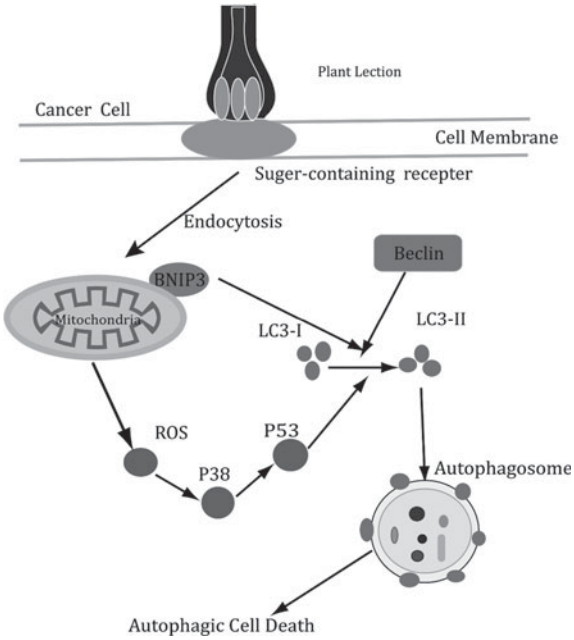

Fig. 49.6 Plant lectin-induced autophagy pathway of ROS-p38-p53

autophagic vesicles were observed. At the same time, ConA has also been shown to act as a T-cell mitogen, which activates the activity of the immune system while inducing autophagy. It promotes the production of cytokines in tumor cells, and then recruits lymphocytes and eventually destroys tumor cells. After performing all these missions, the endocytosed ConA is degraded and digested by lysosomes.

49.3 Conclusion

Many anticancer drugs are powerful inducers of autophagy. Optimists view these as evidence that induction of autophagy is a suitable target for cancer therapy, while pessimists believe that this is a stress response of autophagy against the toxic effects of cancer drugs, both of which have experimental support. However, since the targets of all the current anticancer drugs are different from those of autophagy, it is difficult to analyze the role of autophagy. To quell this debate, we must develop new compounds specifically targeting autophagy, and then we can know how to further transform the understanding of autophagy pathway into a new means of anticancer. Autophagy occurs when anti-tumor drugs act on tumor cells. This is related to the type of drug, the type of tumor cells, the concentration of the drug, and the time of the drug acting on the cells. After autophagy was induced by drugs in tumor cells, two different outcomes would occur, namely, protecting cells from damage caused by the surrounding environment and initiating cell-active type II cell death program. At the same time, this process may also affect the occurrence of apoptosis. The close relation between anti-tumor drugs and factors related to autophagy, as well as autophagy and apoptosis, needs to be further studied. The anti-tumor drugs targeted at the autophagy signaling pathway are highly significant to the molecular treatment of tumors. How to make anti-tumor drugs induce autophagy in tumor cells to cause cell death? How to eliminate the protective effect of autophagy on tumor cells? If these problems are solved, drugs inducing autophagy of tumor cells will play an active role in the treatment of tumors.

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Chapter 50
Progress of Anti-aging Drugs Targeting Autophagy

Jinku Bao, Bo Liu, and Chuanfang Wu

Abstract Senescence is a progressive process of degeneration that occurs when cells and organisms mature. Many studies have shown that autophagy is closely related to senescence. Autophagy gradually decreases with the senescence activity of cells, and vice versa. Therefore, moderate autophagy can protect the body and inhibit cell senescence. The inactivation of genes encoding nematode insulin-like tyrosine kinase receptor (daf-2) inhibited the activity of type I PI3K (age-1), Akt molecules (akt1, akt2), PDK (pdk-1), and TOR, and increased the lifespan and autophagy of *Caenorhabditis elegans*.

Keywords Senescence · Autophagy · Insulin-like/PI3K pathway

50.1 Principles of Autophagy-Related Anti-aging Drugs: Caloric Restriction and Autophagy

50.1.1 Calorie Restriction Relieves Attenuation of Autophagy

Autophagy can promote the degradation of damaged proteins and organelles in cells and enter recycling to prevent the deposition of harmful substances in cells. Regulation of autophagy has been considered as an anti-aging strategy. Caloric restriction (CR) refers to the total amount of calories that limit daily intake when providing sufficient nutrients such as essential amino acids and vitamins to ensure that the organism does not suffer from malnutrition. Calorie restriction plays a key role in anti-aging

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intervention. Both fasting and CR have a salient role in upregulating autophagy markers and autophagy activation. Modulated autophagy can be achieved by both fasting and CR, and plays a crucial role in normal function and homeostasis of cells, leading to an improvement in the health and function of various organs and tissues, muscle, liver, kidney, heart, pancreatic and the nervous system being examples (Bi et al. 2018). To date, most animals including macaques have been tested for life prolonging by caloric restriction. The findings suggest caloric restriction can reduce the incidence of diabetes, cardiovascular disease, cancer, and brain atrophy.

In addition, related studies have also found that caloric restriction can significantly increase the autophagic proteolysis rate of liver cells. Interestingly, caloric restriction induces autophagy, decreases the activity of mTOR, PKA, and PKB/Sch9 signaling, thereby prolonging life in model organisms. Moreover, ATG gene is essential in autophagy, and it also plays a key role in caloric restriction, proving that caloric restriction is closely related to autophagy. For example, autophagy is activated in ATG5 transgenic mice, which is thinner than age-matched mice. Although autophagy regulation declines with age, further studies have found that caloric restriction can effectively delay this declined process. Cavallini et al. believe that the degree of caloric restriction on the autophagy function of the liver depends on the time and extent of the restriction, and is proportional to the life expectancy. Therefore, caloric restriction can delay the decline of age-related autophagy. Calorie restriction is a potent inducer of autophagy.

On the one hand, it prevents the recession of the autophagosome degradation pathway and maintains the body's ability to degrade sirtuin, thereby avoiding the accumulation of damaged proteins in cells; on the other hand, caloric restriction can prevent the aggregation of degenerated mitochondria in aging cells by activating autophagy, and degrade the excess peroxidase in the body, which exerts the anti-aging effect. Recently, many studies have shown that intermittent fasting can exert similar effects as CR (Cerletti et al. 2012; Burkewitz et al. 2016; Cheng et al. 2005). Benefits related to cardiovascular health include protection of heart against ischemic injury, reduced body mass index and blood lipids (Ahmet et al. 2005), improved glucose tolerance (Zuo et al. 2016), and lower incidence of coronary artery disease (Horne et al. 2015). The positive effects of intermittent fasting on brain health in pre-clinical studies comprised improved cognitive function with reduced oxidative stress during middle age when IF was commenced in young adult age (Li et al. 2013).

50.1.2 The Roles of Proteins, Mitochondria, Peroxidases, and Cell Membranes in Caloric Restriction

The ability to degrade proteins gradually decreases with age. The longer a protein lives, the more likely it is to be modified after transcription, and the more likely it

is misfolded. Autophagosome pathway and proteasome pathway are the two main mechanisms of protein degradation. The proteasome pathway primarily degrades short-lived proteins, while the autophagosome pathway primarily degrades long-lived proteins. Almost all of the long-lived proteins, most macromolecules, and all organelles in the cell are transported to the lysosome for degradation by autophagy to satisfy the metabolic needs of cells themselves and the renewal of organelles. A significant reduction in autophagosome's ability to degrade proteins can result in the accumulation of age-related protein carbonyl derivatives. Calorie restriction prevents the decay of the autophagosome degradation pathway to avoid the accumulation of damaged proteins within cell.

Mitochondria are degraded by the autophagy pathway. There are a large number of degenerated mitochondria containing mutated DNA in aging cells, which is caused by decreased function of autophagy with age. Related biological model studies have also demonstrated that decreased autophagy function with age can lead to accumulation of damaged mitochondria. The accumulation of damaged mitochondria and other biological wastes in cells can weaken the autophagy function and make the damage of mitochondria more serious, thus forming a vicious circle. However, caloric restriction can prevent the aggregation of degenerative mitochondria in aging cells by activating autophagy.

The decrease rate of cell peroxidase renewal leads to increase in oxidative stress and a corresponding change in membrane lipid composition with age. In fungi and mammals, peroxidase is degraded by the autophagy pathway, that is, autophagy can selectively degrade certain abnormal peroxidases to protect the body. Autophagy declines with age. Parallel to this process also includes the degree of cell membrane denaturation. Polysterols in the membrane lipids also accumulate with age, which reflects the disorder of free radical metabolism in the cell membrane. Dietary control can significantly reduce the accumulation of polysterols and enhance autophagy.

50.1.3 The Pathway of Calorie Restriction—Calorie Restriction and Diet Control

Diet control may delay senescent and slow down the progression of many senescent diseases. Studies have shown that limiting the food intake of model organisms increases the average and maximum life spans of these species. For example, fasting rodents one day a week may have anti-aging and anti-tumor effects. The anti-aging effects of dietary restriction may be through two different methods: a 30–50% reduction in food consumption or intermittent eating per day. These two methods have similar effects on health and longevity, but have different effects on metabolism.

50.2 Anti-aging Drugs Based on Caloric Restriction and Autophagy

50.2.1 Anti-lipolytic Drugs

The practice of extending life by dieting is common throughout human history, but considering the basic metabolic needs of the human body, it is not advisable to delay aging by blindly eating restrictions. Anti-lipid degradation drugs can mimic the beneficial effects of caloric restriction on autophagy while avoiding the potential adverse effects of caloric restriction on human health. According to the mechanism of autophagy and anti-aging, research on anti-aging-related drugs has great application prospects for delaying aging. At present, some anti-lipolytic drugs are mainly used to reduce blood lipids, such as Acipimox, which can inhibit the release of free fatty acids from whole body fat tissue, reduce the synthesis of cholesterol and triglyceride, and thus reduce the level of total cholesterol, triacyl, and low-density lipoprotein in plasma. At the same time, these drugs also have antioxidant effects, which can inhibit the oxidation of cell membrane lipids and protect cell membranes. In fact, the protection of the cell membrane is equivalent to alleviating the process of cell aging. Anti-lipolytic drugs can mimic calorie restriction, thereby triggering moderate autophagy to alleviate the aging process.

Studies have found that certain natural polysaccharides have similar anti-aging effects. For example, coriolan can be used as a natural drug polysaccharide to acetylate mouse peritoneal macrophages. It can also increase the number of low-density lipoprotein (acLDL) receptors, thereby promoting the binding, internal migration, and degradation of macrophages and acLDL. The incidence of experimental hyperlipidemia and atherosclerotic plaque was significantly reduced, and the plaque area was significantly reduced. Intraperitoneal injection of sugarcane polysaccharides in rats with long-term high-sugar diet resulted in a significant decrease in blood high-density lipoprotein cholesterol (HDL-C), lipid peroxide (LPO), aspartate aminotransferase (GOT), and GPT, while liver phospholipids increased significantly.

In addition, studies on the anti-aging pharmacological mechanism of Lycium barbarum polysaccharides have also found that it has a hypolipidemic effect. In fact, many natural Chinese herbal medicines with anti-aging effects, such as astragalus, salvia miltiorrhiza, ginseng saponins, have the effect of anti-oxidation, scavenge free radicals, lowering blood lipids, and preventing peroxidation.

50.2.2 Rapamycin

After treatment of eukaryotic cells with rapamycin, a nutrient-deficient state can be produced and autophagy can be activated. Therefore, long-term administration of an appropriate amount of rapamycin to a mammal can stimulate autophagy by blocking the mTOR pathway, thereby achieving a calorie-restricted anti-aging effect

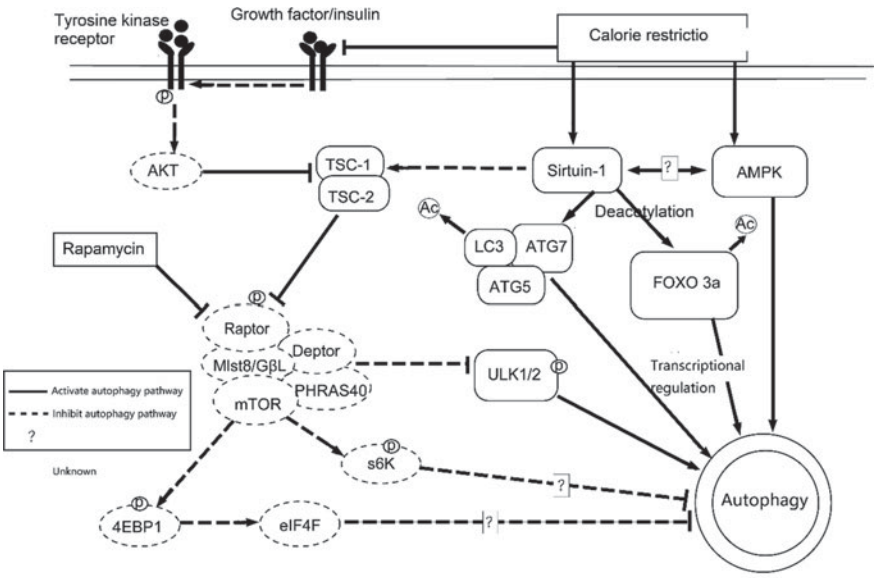

Fig. 50.1 The relationship between caloric restriction and autophagy and aging

(Fig. 50.1). Rapamycin has broad prospects as an autophagy-inducing drug, but its side effects have limited its use as an anti-aging drug. The metabolic disorder of reactive oxygen species is a major factor in promoting aging, and the oxidative action of rapamycin will undoubtedly make this process worse. Therefore, the inclusion of rapamycin in anti-aging autophagy-inducing drugs may have more harm than good, and finally counterproductive.

50.2.3 Spermidine

Recent studies have found that exogenous spermidine can prolong the lifespan of aging animals by modifying and inducing autophagy and inhibiting cell necrosis (LaRocca et al. 2013). The researchers applied spermidine to the aged yeast cells with reduced levels of endogenous polyamine synthesis and found that supply of exogenous spermidine significantly increased the lifespan of wild-type BY4741 yeast cells. It was four times longer than cells treated without spermidine. Similar results were obtained for the wild-type DBY746 yeast test. Moreover, exogenous spermidine can also lead to a steady increase in the amount of endogenous spermidine synthesis in senescent cells. The researchers also found that yeast cells treated with spermidine showed greater resistance to heat and H_2O_2 oxidation. The study is not limited to yeast. In the study of multicellular organisms, researchers added the right amount of spermidine to the food of fruit flies. The results showed that the average lifespan

of flies fed with this substance increased by 30% compared to the flies fed without spermidine, and the endogenous spermidine levels were also increased by about 20%. The results were also confirmed in human peripheral blood mononuclear cells. After 12 days of treatment with spermidine, the cell viability was more than three times higher than that of cells without spermidine. The rate of cell necrosis was significantly reduced by treatment of spermidine. Spermidine acts as inhibitor of histone acetyltransferase, reduces histone acetylation and upregulates ATG gene expression, and induces autophagy and prolongs lifespan. It has demonstrated that spermidine can be used as an autophagy inducer to delay aging.

50.2.4 Natural Medicines

In addition to spermidine, recent studies have found that resveratrol can also regulate autophagy to delay aging (Shen et al. 2017). Resveratrol can directly bind to androgen receptor molecular targets, thereby inhibiting the PI3K/Akt pathway (Jiang et al. 2009), inducing mitochondrial release of cytochrome c from tumor cells, forming an apoptosis complex, causing caspase9 cleavage, and ultimately inducing caspase-independent autophagic cell death. Studies have shown that resveratrol can allosterically activate the NAD+ dependent histone deacetylase Sirtuin 1. The protein is highly conserved in evolution and has been shown to be a protein that extends the lifespan of yeast. In addition, some natural Chinese herbal medicines or their combination drugs have certain regulatory effects on aging-related genes. Studies have shown that *Polygonum multiflorum* can reduce the expression of p53 gene in aged mice (Androutsopoulos et al. 2011).

50.3 Conclusion

A large number of biochemical and genetic evidences suggest that autophagy and its signal-regulating systems play a key role in the anti-aging system, and the crosstalk between their key signaling molecules can serve as a breakthrough in the development of anti-aging autophagy-inducing drugs. Studies have shown that the activities of age-1, AKT1, AKT2, PDK, and mTOR were inhibited when the nematode insulin-like tyrosine kinase receptor gene undergoes a functional inactivating mutation, and the lifespan of *Caenorhabditis elegans* was prolonged. In the downstream of this signaling pathway, type I PI3K converts phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-diphosphate to phosphatidylinositol-3,4-diphosphate and phosphatidylinositol-3,4,5-triphosphate. They in turn bind to the platelet leukocyte C kinase substrate homology domain of Akt and its activator PDK1, respectively. Activated Akt and PDK1 phosphorylate other protein kinases including the autophagy inhibitor mTOR. The above evidence shows that the encoded product of

this gene is likely to be a key molecule in connection with cellular senescence and autophagy regulation.

PTEN is a phosphatase encoded by a tumor suppressor gene that dephosphorylates the 3' end of the type I PI3K product, thereby downregulating the PI3K/ATG cascade. Studies have shown that this enzyme is also associated with autophagy and aging signaling pathways. Mutations in Daf-2 and ATG-1 can prolong the lifespan of nematodes, but this effect also disappears when the PTEN homolog of the nematode (daf-18) is mutated. In the cloned human colon cancer cell experiment, it was observed that type I PI3K and Akt inhibited autophagy, and PTEN also activated autophagy. Therefore, autophagy-promoting factors can be used for longevity in *C. elegans* and mammalian cells. The same results were also found in studies of other animals, which demonstrate that autophagy plays a very important role in delaying aging.

Various animal models have been used to study autophagy and anti-aging mechanisms or the development of anti-aging drugs. The study of the model organism "nematode" provides the first genetic evidence for this connection between autophagy and longevity. If the mechanism of autophagy and aging is clarified by biological models such as yeast, nematodes, and fruit flies, then the development of autophagy anti-aging drugs must be inseparable from mammalian models that are more closely related to humans. A variety of mutant mouse models are currently widely used to provide a broader basis for autophagy and anti-aging mechanisms and drug development research. For example, using the autophagy-injured mouse model, the variation of "autophagy protease level random body age growth" was obtained. Through in-depth study of hepatocytes cultured in vivo and in vitro, the gradual decrease of autophagy activity was inferred. The role played by functional decay of old body. However, the current research on the mechanism of autophagy and anti-aging is limited to mice or some lower organisms. Even in the experiments, human cells cultured in vitro are used. However, the mechanism of anti-aging autophagy drugs remains to be further studied, because the complexity of the human physiological environment is far beyond our imagination. Therefore, whether these autophagy-inducing drugs that exert anti-aging effects in animal models can be truly used in human clinical treatment is still unknown. Further activity and toxicological testing for these drugs is also required. However, from the perspective of future development, we may boldly predict that such drugs might have broad application prospects.

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Chapter 51
Drug Development and Treatment of Autophagy in Other Diseases

Jinku Bao, Bo Liu, and Chuanfang Wu

Abstract In addition to tumors and aging that are associated with autophagy, many other diseases are also regulated by autophagy, including liver disease, myopathy, immune pathogen infection, cardiovascular disease, and so on. This chapter will detail the relationship between autophagy and these diseases and their underlying molecular mechanisms. We summarized the current research status of autophagy as a target for the treatment of related diseases, and prospected the development of related drugs and therapeutic strategies. We hope to provide new ideas for finding new therapeutic targets through the autophagic signaling pathways.

Keywords Autophagy · Liver disease · Myopathy · Immune pathogen infection · Cardiovascular disease

The previous parts provide details about the relationship between autophagy and tumors and aging, and briefly describe the research progress in the treatment of these diseases by regulating autophagy. In addition to the abovementioned diseases associated with autophagy, the regulation of autophagy is also important for other diseases. Herein, we briefly introduce the relationships between autophagy and other diseases and also prospects for the development of related drugs.

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51.1 Neurodegenerative Disease

Neurodegenerative diseases may be caused by concurrent defects in the autophagy pathways. Targeting autophagic signaling pathways to find new therapeutic targets, and appropriate activation of autophagy may be beneficial for the treatment of neurodegenerative diseases. For most of the neurodegenerative disorders discussed earlier, the available evidence favors a strategy of enhancing the efficacy of autophagy by targeting the stages that are specifically disrupted in each disease. The expected benefits of such modulation might include lowered amounts of toxic protein aggregates and incompletely digested autolysosomal metabolites, more effective responses to stress from redeploying non-essential constituents for energy and adaptive protein synthesis, and suppression of apoptotic cascades or necrosis by prevention of lysosomal membrane destabilization. Preventing or reversing impairments in autolysosomal clearance of substrates is a promising therapeutic approach and may prove to be the strategy of choice or necessity for certain neurodegenerative disorders. At least in cell models, when lysosomal clearance is impaired, inducing autophagy exacerbates pathology, which implies that, in a disease such as Alzheimer's disease, the success of any autophagy induction intervention may depend on first relieving the block in lysosomal clearance or possibly preempting this block by stimulating autophagy induction very early in the disease (Nixon 2013).

TOR has become a common autophagy target for the treatment of neurodegenerative diseases. Rapamycin has been used clinically to alleviate neurodegenerative diseases. Therefore, the development of a drug similar to rapamycin, which has the same function and lower toxic side effects, can continue to be a direction for the development of new drugs. In addition, based on the pathogenesis of some autophagic neurodegenerative diseases shown in Table 51.1, we can seek some new drug development directions. For example, autophagy caused by mutations in dynein (DCTN1) is blocked, and drugs that can replace DCTN1 and have low toxic side effects are sought.

51.2 Liver Disease

It is important to distinguish the level of autophagy in different liver diseases and its effects. In liver diseases, autophagy has both a retarding effect and a promoting effect. Increasing the levels of autophagy can improve the condition in liver ischemia–reperfusion injury, alcoholic liver disease, non-alcoholic fatty liver disease, and primary liver cancer. Autophagy can promote the replication of HBV and HCV in hepatitis B and C virus. However, the mechanism by which autophagy causes the abovementioned diseases is not fully understood.

It has been demonstrated that autophagy provides necessary energy to initiate and maintain the activated phenotype of hepatic stellate cells (HSC) by degrading lipid droplets after injury, suggesting that autophagy may be promoted by tissue fibers in

Table 51.1 Common databases of protein interaction information

Database	Describe
HPRD (human protein reference database) http://www.hprd.org	The HPRD database is a comprehensive database of protein interactions, protein annotations, domains, post-transcriptional modifications, subcellular localization, enzyme–substrate relationships, tissue expression, disease-related information
DIP (database of interacting proteins) https://dip.doe-mbi.ucla.edu/dip/Main.cgi	The DIPTM database catalogs experimentally determined interactions between proteins. It combines information from a variety of sources to create a single, consistent set of protein–protein interactions
IntAct (molecular interaction database) http://www.ebi.ac.uk	The IntAct database is a public database for storing and analyzing interactions between biomolecules. The database mainly records binary interactions. The database records the experimental methods, experimental conditions, and interaction domains in detail, including human, yeast, fruit fly, E. coli, and other species. Its web interface provides a textual description and graphical description of protein interactions, and allows browsing of interaction networks in the context of Gene Ontology (GO) annotations of interacting proteins
BioGrid (database of protein and genetic interaction) http://thebiogrid.org/	BioGRID is an interaction repository with data compiled through comprehensive curation efforts. Our current index is version 3.5.170 and searches 68,754 publications for 1,670,339 protein and genetic interactions, 28,093 chemical associations, and 726,378 post-translational modifications from major model organism species. All data are freely provided via our search index and available for download in standardized formats
MINT (molecular interaction database) http://mentha.uniroma2.it/	MINT database mainly stores protein physical interactions, with particular emphasis on mammalian protein interactions, as well as protein interactions of some yeasts, fruit flies, and viruses
Mentha http://mentha.uniroma2.it/	Mentha collects evidence from different sources and presents the data in a complete and comprehensive manner. The data comes from a database of corrected protein–protein interactions that have joined the IMEx Alliance. It provides a number of tools to analyze specific proteins in an interaction network. The database also stores protein–protein interaction information in published papers

(continued)

Table 51.1 (continued)

Database	Describe
STRING https://string-db.org/cgi/input.pl	STRING is a database of known and predicted protein–protein interactions. The interactions include direct (physical) and indirect (functional) associations; they stem from computational prediction, from knowledge transfer between organisms, and from interactions aggregated from other (primary) databases
SIGNOR (signaling network open resource) https://signor.uniroma2.it/	SIGNOR, the signaling network open resource, organizes and stores in a structured format signaling information published in the scientific literature

fibrogenic cells (Virginia and Friedman 2012). Therefore, inhibition of autophagy can inhibit the activation of HSC and reduce the extent of liver fibrosis. In addition, inhibition of autophagy can also reduce HBV or HCV replication.

Tissue-specific knockout experiments in mice indicated that hepatocyte autophagy plays an extremely important role in the quality control of intracellular proteins and organelles (Beth and Guido 2008). It may play an important role in the pathogenesis of α1-antitrypsin deficiency, the most common hereditary liver disease in humans. The occurrence of this disease may be similar to a neurodegenerative disease caused by agglomerated proteins, and the pharmacological activity of autophagy should be able to function in this environment. Functional point mutations in α1-antitrypsin (α1-AT) can prevent normal protein folding and allow normal proteins secreted by the liver to form aggregated complexes in the endoplasmic reticulum. Wild-type α1-antitrypsin is mainly degraded by protease, and mutant α1-AT (α1-ATZ) is mainly degraded by autophagy. In the cell line lacking Atg5, the degradation of the insoluble mutant α1-ATZ is reduced, resulting in accumulation of cytoplasmic inclusion bodies. Furthermore, the transgenically expressed mutant α1-ATZ is sufficient to induce autophagy in mouse hepatocytes in vivo. The exact toxic effects of α1-ATZ in liver cells are still unclear. Interestingly, aggregative proteins are capable of blocking autophagic proteins leading to a reduction in hepatocyte autophagy (and its cytoprotective and anti-cancer effects). Selective promotion or inhibition of autophagy according to different causes may become a new means for treating liver diseases. While autophagy that specifically blocks fibrotic cells may be a new strategy for anti-fibrotic therapy, the current inhibition of autophagy is still a challenge.

51.3 Myopathy

It is well known that lysosomes are membrane-linked acidic organelles associated with autophagy-degrading macromolecules. In myopathy, the lysosomal structure is abnormal, as well as the occurrence of functional damage, resulting in the accumulation of autophagic vacuoles in the muscle fibers. This may be the reason why the accumulation of autophagic vacuoles leads to the development of related myopathy and causes its pathological process. The pathogenesis of critical illness myopathy (CIM) may not be induced by the caspase-3-dependent apoptotic pathways or the PARP-dependent apoptosis pathways. It is reported that experimental results suggested that pULK may induce autophagy in myocyte cells, thus causing autophagic cell death (Barnes et al. 2015). However, the molecular mechanism by which pULK activates the downstream pathway in CIM is still unclear. The cells can be degraded by the "ubiquitin-p62-LC3-autophagosome" pathway, and autophagy remains the main cell degradation mode of CIM. Therefore, abnormal regulation of autophagy in myopathy is considered to be the main pathological mechanism of disease development. In addition, lysosomal autophagic myopathy is mainly caused by the lack of lysosomes. Accumulating evidence indicated that rapamycin ameliorated the clinical and biochemical phenotype of mouse, worm, and cellular models of mitochondrial disease via coordinated activation of autophagy and lysosomal biogenesis (Civiletto et al. 2018).

51.4 Immune Pathogen Infection

Autophagy is involved in the process by which certain endogenous synthetic microorganisms deliver antigen to MHC-II (e.g., Epstein–Barr virus antigen), resulting in activation of CD4 + T lymphocytes. Other viral antigens (such as influenza virus matrix proteins) can be fused with LC3 to selectively target autophagosomes, thereby enhancing the marked enhancement of CD4 + T lymphocyte responses (Fig. 51.1). The importance of autophagic microbial antigen delivery in adaptive immune processes is unclear. However, anchoring autophagy-mediated proteins to the MHC-II loading chamber may increase the effective pathway for T helper cell responses, thereby increasing the efficacy of the vaccine. Previous studies have found that surviving intracellular microorganisms against autophagy-activated pathways or membrane transport events required for lysosomal delivery and degradation by means of directly or indirectly inhibiting their interaction with autophagy proteins (Jo et al. 2013). Pathogenic microorganisms can counteract or even counteract autoimmunity of the host by producing virulence factors. By selectively disrupting the interaction between microbial virulence factors and their targeted autophagy proteins, host cells can protect against foreign microbes by regulating autophagy, which should be considered as a novel antibacterial therapeutic strategy.

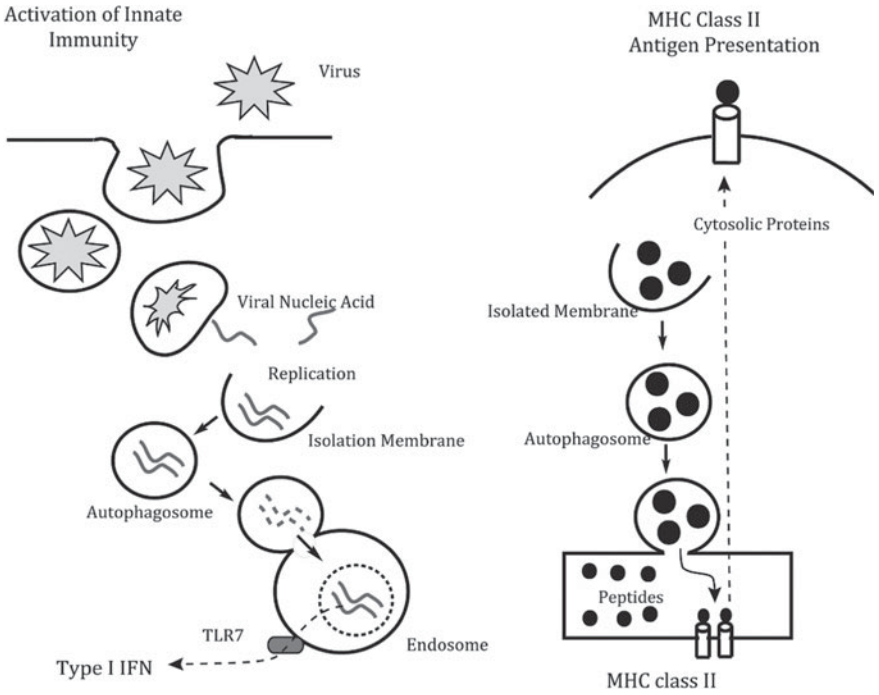

Fig. 51.1 Autoimmune and adaptive immune-related autophagy

51.5 Cardiovascular Diseases

Autophagy is an important homeostatic mechanism that maintains normal cardiovascular morphology and functions under normal conditions. However, autophagy can also cause many cardiovascular-related diseases, such as cardiac hypertrophy and heart failure. Current studies have shown that most of the drugs for treating cardiovascular-related diseases showed inhibition of autophagy. Numerous studies have shown that it is promising to treat or prevent some cardiovascular diseases by regulating the autophagy pathways. For example, 3-MA, an inhibitor of PI3K, can attenuate or inhibit the development of autophagy to treat cardiovascular disease for its therapeutic purposes.

Verapamil has been used as an L-type calcium channel blocker for hypertension, angina, and arrhythmia for more than 30 years. It has recently been found to induce autophagy and prevent vascular smooth muscle cell proliferation (Salabei et al. 2012). The mechanism is independent of blocking calcium channels. This finding may be applied to more effectively prevent AS and vascular restenosis. Simvastatin has recently been found to enhance autophagy and mitochondrial degradation in mouse hearts by inhibiting m TOR (Andres et al. 2014). This effect is directly related to inhibition of HMG-Co A reductase and inactivation of Akt, which in turn lowers

cholesterol levels. Simvastatin also reduces infarct size during myocardial infarction through a mechanism of mitochondrial autophagy. Treatment of diabetic mice with metformin can reduce cardiomyocyte apoptosis by inducing autophagy and prevent the development of diabetic cardiomyopathy. In addition, long-term use of statins can prevent the occurrence of coronary artery disease, reduce the incidence of fatal and non-fatal cardiovascular events, limit the scope and severity of cardiovascular events, and have a protective effect on the heart. Statins can intervene in multiple aspects of the pathological process of atherosclerosis to achieve the following goals: stabilize atherosclerotic plaque, reduce plaque volume, delay the progression of atherosclerosis, and reduce the occurrence of cardiovascular events.

It is speculated atorvastatin, a hypolipidemic drug, can protect vascular endothelial cells by affecting autophagy induced by damage to vascular endothelial cells. Some results show that atorvastatin can reduce the occurrence of autophagy and protect blood vessels in cardiovascular disease. In addition, in the early stage of damage, the cells degrade most of their long half-life proteins by autophagy, so that the cells can obtain amino acids and other macromolecular substances for biosynthesis to protect cells. Therefore, the early use of atorvastatin to play its role in inhibiting autophagy to protect endothelial function is particularly critical.

51.6 Kidney Disease

Glomerular mesangial cells undergo proliferation, proliferation, contraction, and abnormal apoptosis after injury, which can cause glomerular hemodynamic changes. This phenomenon is closely related to nodular diabetic glomerulosclerosis, amyloid glomerulopathy, fibronectin nephropathy, renal fibrosis, and other diseases. TGF-β1 induces apoptosis in mesangial cells with high expression of LC3, which induces autophagy. However, after knocking out or silencing autophagy-related genes, the protective effect of TGF-β1 on mesangial cells was reduced. Autophagy regulates the pathological processes associated with renal fibrosis and plays a protective role in mesangial cell apoptosis. Therefore, TGF-β1 may become a drug target, thus providing a new direction for drug development.

The Chinese medicine rhubarb has been confirmed to have a definite renal protective effect (Cao et al. 2017). Its main active ingredients are emodin, which has anti-tumor, anti-oxidation, and anti-inflammatory effects. And it regulates pharmacological effects such as lipid metabolism and anti-renal fibrosis. A bulk of studies have shown that emodin can inhibit the expression of LC3-II and autophagy in renal tubular epithelial cells induced by Hank's balanced salt solution (HBSS) by regulating mTOR signaling pathways. Rhein can also inhibit the expression of autophagy protein in renal tubular epithelial cells induced by HBSS starvation by regulating the activity of mTOR signaling pathway.

In glomerular endothelial cell autophagy, the mammalian target of rapamycin (mTOR) is the central link in autophagy regulation. Rapamycin can regulate the activation of Atg13 and ULK1 (Atg1 homolog of yeast) by inhibiting the activity

of mTOR, thereby regulating the formation of ULK1 complex and inducing the occurrence of autophagy.

Cisplatin is highly regarded as a classic nephrotoxic drug. In the RTEC injury model induced by cisplatin, increased autophagy-based markers such as LC3, Beclin1, and Atg5 have been observed (Herzog et al. 2012). It was also found that autophagy inhibitors inactivated Beclin1 and Atg5 and enhanced caspases activity and promoted RTEC apoptosis. Conversely, increasing autophagy levels inhibit RTEC apoptosis. Inhibition of autophagy or knockdown of the shRNA fragment of the autophagy gene Beclin1 increases RTEC apoptosis. Therefore, autophagy may be a protective mechanism involved in the potential cryoprotection. In summary, autophagy may play a protective role in toxic kidney injury, and its protective mechanism needs to be further clarified.

51.7 Hepatic Encephalopathy

In astrocytes, overexpression of the mTOR signaling pathway caused by point mutations in the tumor suppressor gene TSC1 leads to cell enlargement. Although this view has not been confirmed in depth, this finding provides new ideas for understanding the role of ammonia toxicity in the pathogenesis of hepatic encephalopathy. Increasing intracellular glutamine (produced by ammonia) not only causes brain swelling and enlargement through intracranial pressure, but also causes osmosis, which increases the volume of astrocytes in a short period of time and will take a long time. This may result in inhibition of autophagy, which in turn leads to the elimination of stellate cells, which are inhibited by abnormal cellular structures such as mitochondria. As discussed above, due to the complicated roles of autophagy in these diseases, inducing or inhibiting autophagy as the therapeutic method for such diseases depends on various factors including the progression of the disease and the nature of the treatment.

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Chapter 52
Systems Biology Approaches in Autophagy Research

Jinku Bao, Bo Liu, and Chuanfang Wu

Abstract As a classical form of programmed cell death, autophagy is widely involved in cellular metabolism and vital for the maintenance of homeostasis in physiological and pathological states. With multiple levels of regulation and signaling integrated in, autography presents complicated relevance with various diseases, such as cancer and neurological diseases. The emerging subject, systems biology, along with multi-omics approaches, offers a new strategy to investigate these interactive processes from a holistic perspective. In this chapter, we focus on the systems biology method for autophagy research and introduce essential research skills and procedures. The critical step of systematic study is to explore interplay between biological molecules based on massive biological data, which requires construction of networks in different biological levels, modification, and identification of key pathways and targets via optimized algorithm and experimental verification. Guided by systems biology research, drug design can thus be strengthened by efficient screening and accurate evaluation. Overall, systems biology promises to act as a powerful tool which both helps to clarify the profound mechanism and to develop efficacious medicine.

Keywords Autophagy · Systems biology · Multi-omics · Interaction network

It has been demonstrated that autophagy is an important metabolic process that plays an important role in maintaining homeostasis. More and more studies have found that autophagy is closely related to a variety of diseases, including neurodegenerative diseases, infectious diseases, cardiovascular diseases, and tumors (Choi et al. 2018; Lee et al. 2016; Lorente et al. 2018; Xie et al. 2011). These connections are derived

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from the different roles that autophagy plays in different diseases or the different stages of diseases. For example, autophagy can reduce the accumulation of toxic proteins (e.g., misfolded proteins) in cells, thereby effectively protecting nerve cells and preventing the occurrence of neurodegenerative diseases. Autophagy also plays an important role in the differentiation of neutrophils in vivo. The immune cells are mediators of innate immunity and participate in immune responses. Specific knockout of autophagy-related (ATG) genes in mice causes cardiovascular disorders. In different stages of tumorigenesis, autophagy is more like a "double-edged sword." For example, in hepatocellular carcinoma, researchers have found that enhanced autophagy can effectively inhibit the development of the cancer, but some researchers have found that obstruction of autophagy can promote chemotherapy-induced apoptotic cell death of cancer cells (White and DiPaola 2009).

Autophagy also plays an important role in the occurrence and development of diseases. It also suggests that effective regulation of autophagy can alleviate the symptoms of the disease and even cure the disease. Therefore, scientists are trying to study the relationship between autophagy and related diseases, and to explore how to maximize drug efficiency through autophagy regulation (Sridhar et al. 2012). The research on the relationships between autophagy and diseases and drug development is not limited to traditional biological methods. With the development of science and technology, more and more new methods based on macroscopic analysis have emerged. This is very important for the use of systems biology to study autophagy and drug development. The following part is a brief introduction to new methods and techniques for studying the relationship between autophagy and disease, and the development of drugs based on system biology methods in autophagy researches.

Systems biology is the study of the composition of all components (genes, nucleic acids, proteins, etc.) in a biological system, the interrelationship of these components under specific conditions, and the establishment of a mathematical model through computational biology to quantitatively describe and predict their function, phenotype, and behavior (Bruggeman and Westerhoff 2007). Modern research has fully recognized that every life process is accompanied by a complex regulatory network, genetic mutations, epigenetic changes, gene transcription, and abnormal protein levels, which have negligible impact on life processes. For example, autophagy involves multiple signaling pathways, such as TOR, Ras-cAMP-PKA, etc. In addition, epigenetic and transcriptional regulations are also closely related to the metabolic process of autophagy (Ng 2010). Similarly, the tumorigenesis is also a complex process involving multiple factors and multiple steps, and tumor is a type of systemic metabolic disorders (Seton-Rogers 2016). With the development of omics technology and the continuous improvement of corresponding analytical techniques, multi-omics methods based on the concept of system biology began to be used by more and more researchers to study the process of disease, and researchers have achieved fruitful results (Hasin et al. 2017).

The so-called multi-omics method integrates various types of omics data, such as genomes, transcriptomes, proteomes, or metabolomes, to systematically analyze and explore the biological significance in biological samples. The adoption of multi-omics methods not only promotes the study of disease mechanisms and the discovery

of pathogenic targets, but also provides new ideas for basic disease science and precision medical research (Li et al. 2017). The key is to construct an interaction network corresponding to biological phenomena based on different biological dimensions (genes, proteins, etc.), including gene–gene interaction networks and protein–protein interaction (PPI) networks. Mathematical models can be used to clarify the relationship between various factors and to identify pathogenic target in these networks. The following example of autophagy and tumors illustrates the construction of a PPI network using systematic biological methods. From a systems biology perspective, tumors are caused by malfunctions induced by abnormal interactions between intracellular components. Tumors are closely linked to biological phenomena such as autophagy. Therefore, we can identify important signal transduction pathways and core proteins by comparing normal interaction network with abnormal networks related to autophagy in tumors. These important signaling pathways and core proteins are valuable drug targets (Li et al. 2017). Targeted therapies can then be created to rectify tumor-associated signaling pathways to achieve the goal of alleviating or curing cancers. Screening targets from these signaling pathways and core proteins is a starting point for drug design, and this can regulate tumor-related signaling pathways to achieve the purpose of relieving or curing tumors.

A brief process for studying the relationship between autophagy and tumor using systematic biology methods is as follows:

1. Initialization of autophagy-related PPI network: collecting corresponding data from public database or using experimental approaches such as yeast two-hybrid system (Y2H) (Hamdi and Colas 2012), affinity purification-mass spectrometry (AP/MS) (Chen and Gingras 2007), and mammalian membrane two-hybrid assay (MaMTH) (Petschnigg et al. 2014) to construct the initial network of autophagy-associated PPI in tumor and normal cells. Several common databases of protein interaction information are listed in Table 52.1 and the constructed network is shown in Fig. 52.1.
2. Modification of PPI network: Integrating high-throughput datasets with relevant mathematical models, including support vector machine (SVM) (Ben-Hur and Weston 2010), hidden Markov model (HMM) (Blunsom 2004), and Bayesian model (Raftery 1995), to remove false positive PPIs from the initial PPI network to get a network read for subsequent use (Fig. 52.1). The information here includes interolog, expression, and domain–domain interaction.
3. Identification of important pathways and core proteins: comparing the expression data of autophagy-related proteins in tumor and normal cells and identifying the topological properties of the network, thereby providing a new molecular basis for important pathways and core proteins related to autophagy (Fig. 52.1). It is worthy of note that protein structure information and phylogenetic analysis could facilitate the identification of important pathways and core proteins.
4. Experimental verification of important pathways and core proteins. Many achievements have been made in the study of autophagy and disease using systems biology. Systems biology is also expanding, and sub-disciplines derived

Table 52.1 Drug development platforms based on systems biology

Database	Homepage	Description
BioMap (Kunkel et al. 2004)	www.bioseekinc.com	The platform can be used to determine the activity of the compound in the early stage of drug development and the determination of effective targets, lead optimization, evaluation of late clinical drug candidates, and predict the mechanism of action of the compound
Connectivity Map (Lamb et al. 2006)	https://clue.io/	A research platform developed by a number of research institutes, including the MIT and Harvard University. The platform concerns functional interrelationships between drugs and diseases. Its purpose is to provide a systematic solution to discover the connections hidden between drugs, genes, and diseases
Ingenuity Pathway Analysis (Krämer et al. 2014)	http://www.ingenuity.com/	Developed by Ingenuity Systems, a professional bio-information platform provider in the United States. It claims to be the world's most complete and accurate database of commercial bio-network pathways and analysis platform
Physiolab Platform (Rullmann et al. 2005)	http://www.entelos.com/index.php	A large-scale dynamic computer simulation disease system analysis platform developed by Entelos Biomedical Company

from systems biology continue to emerge, such as Systems Neurobiology (Gallinat et al. 2007), Systems Biology for Genetic Networks (Cuccato et al. 2009), Systems Biology for Signal Transduction (Klingmüller et al. 2006), Metabolomics-based Systems Biology (Van Der Greef et al. 2006), Systems Developmental Biology (Hall 2012), Cancer Systems Biology (Kreeger and Lauffenburger 2010), Cardiac Systems Biology (McCulloch and Paternostro 2005), and Systems Medicine (Auffray et al. 2009).

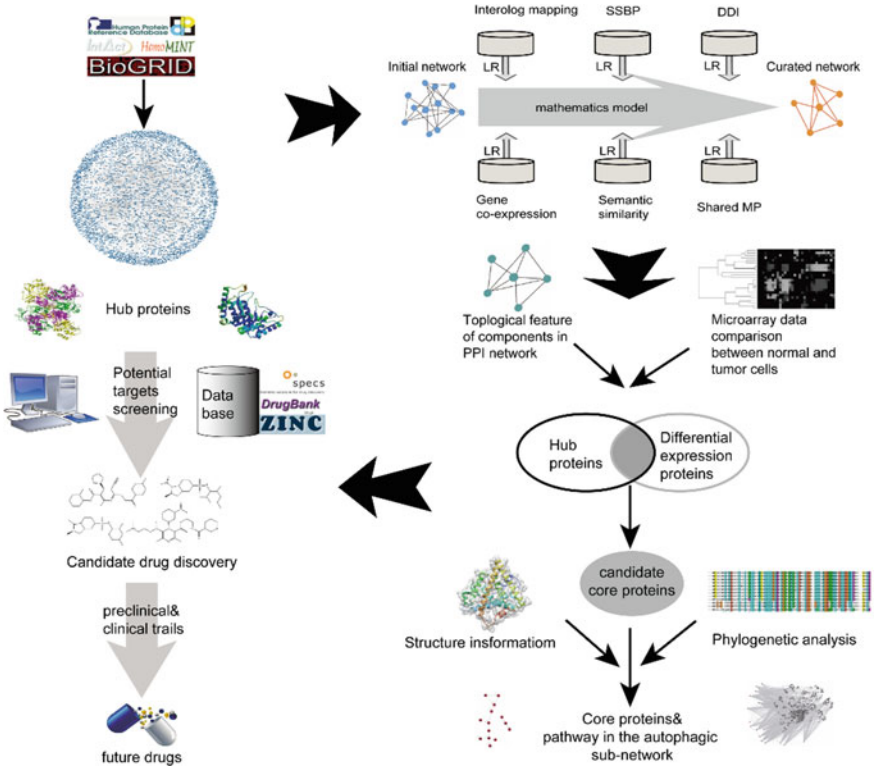

Fig. 52.1 Schematic workflow of developing autophagy-related drug based on systems biology methods

5. Application in drug development. The emergence of systems biology has provided a new impetus for drug development (Butcher et al. 2004). Traditional drug discovery research methods are characterized by low efficiency, slow speed, and low success rate. They are the bottleneck of drug discovery based on single drug target. Systematic biology analyzes multiple factors related to disease occurrence as a whole, thus enabling to gain deeper insight into the mechanism of disease occurrence and to provide constructive guideline for drug development. It can be helpful to tackle the following challenges in drug development: rational drug target identification, confirmation and avoidance of adverse effects before clinical trials, monitoring clinical efficacy, and shortening the development cycle of new drugs. In drug development, mathematical simulation models based on drug-gene-disease data have led to the emergence of drug discovery platforms based on systems biology (Table 52.1). These platforms have become important tools for drug development. At present, most international pharmaceutical companies have successfully applied the systems biology-based drug development platform in drug target identification and evaluation, pharmacological and toxicological

evaluations, efficacy evaluation, and adverse prediction. Encouragingly, pharmaceutical companies begin to gain benefits from systems biology-based drug development platforms, through which they can learn whether new drug products have development value, and also consume less cost and time to develop new drugs that can be marketed.

In summary, systems biology provides new strategies for autophagy research and drug development (Kitano 2002). Although the relationship between autophagy and various diseases has become clearer and the related drug interventions have achieved remarkable results, autophagy inducers currently used in clinical practice are still rare (Levine and Kroemer 2008). This may result from the fact that the mechanisms involved in autophagy-induced disease have not yet been elucidated very clearly. System biology can help to integrate information on various autophagy and disease, and introduce potential drugs for molecular target exploration. It greatly enhances the comprehensive utilization of various information compared with traditional analytical methods and avoids some tedious testing process. It is expected to greatly shorten the cycle of drug development, thus providing powerful assistance for drug development.

52.1 Conclusions

Autophagy is a basic form of physiological regulation and is involved in a variety of physiological and pathological processes (Mizushima 2007). It is closely related to the occurrence and development of tumors, neurodegenerative diseases, aging, and cardiovascular diseases (Mizushima et al. 2008). At present, the relationship between autophagy and disease has become a hot research field (Sridhar et al. 2012). Autophagy can promote the occurrence of tumors in a certain period of time, and it may inhibit the occurrence of tumors in another life process. Autophagy is also closely related to the recurrence of cured primary tumors. Therefore, the development of anti-cancer drugs for targeting autophagy is more complicated than others, and the treatment procedure should be more sophisticated and specific (Degenhardt et al. 2006). At present, drugs, such as rapamycin, lithium salt, and statins, have been developed for the treatment of tumors, neurodegenerative diseases, and cardiovascular diseases (Rahman and Rhim 2017). These drugs regulate autophagy of cells. However, the molecular mechanism of the link between autophagy and various diseases remains to be revealed. Therefore, with the deepening of the understanding of autophagy, we need to comprehensively analyze the relationship between autophagy and various diseases and find out key proteins and important signaling pathways between autophagy and disease. Further, drug development can be carried out for the identified key proteins and important signaling pathways, thereby achieving the purpose of treating diseases by regulating autophagy. Systems biology provides a promising strategy for drug development for the treatment of various autophagy-related diseases.

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Part XIII
Natural Products on Regulation of Autophagy

There are two types of degradation systems in eukaryotes, lysosome, and proteasome system. For only recognizing the ubiquitylated substrates, proteasome system presents higher selectivity than macroautophagy (all the mentioned autophagy in this chapter signifies macroautophagy) system, which is a degradation system without specific recognition function but take charge of degrading most of the big molecules and organelles. Thus, macroautophagy system plays an essential role in maintaining cellular homeostasis (Zhan 2018). In 2016, Japanese physiologist Yoshinori Ohsumi was awarded the Nobel Prize of Physiology and Medicine for his outstanding contribution on revealing the mechanism of autophagy.

Many natural products derived from plants are found in our daily diet. A number of studies have demonstrated that the inhibition of disease by natural products is not only reflected in the regulation of apoptosis, but also reversed the progression of the disease by regulating the autophagy process. They may work through classic (with Berlin 1 mediated) or nonclassic (without Berlin 1 mediated) autophagic pathways to militate against pathological processes. The role of natural products in regulating autophagy may be used as a primary mechanism of action for the prevention and treatment of some diseases, or as a means of adjuvant therapy. Research and development of these drugs not only provide a basis for the prevention and treatment of some complex diseases, such as tumors, neurodegenerative diseases, cardiovascular diseases, metabolic diseases, and immune system diseases, but also contribute to understanding the molecular mechanism of autophagy. In this chapter, we focus on the study of natural products on cancer, neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease, as well as cardiovascular diseases.

It must be mentioned that there may be contradictory reports on many natural products' effects on autophagy. One of the reasons is that the role of these natural products depends on the dose or concentration of the drug used. This phenomenon is called the hormesis effect (Wang 2018), which could explain the different effects of one similar drug. According to the definition of toxic excitatory effects, low doses of drugs often have protective effects, while higher doses may produce cytotoxic or inhibitory effects. The natural products' regulation often also exhibits this pattern.

The natural products derived from plants mainly include polyphenols (polyphenolic compounds have flavonoid structure), alkaloids, polysaccharides, volatile oils, terpenoids, lignans, coumarins, sap The natural products derived from plants mainly include polyphenols (polyphenolic compounds have flavonoid structure), alkaloids, polysaccharides, volatile oils, terpenoids, lignans, coumarins, saponins, cardiac glycosides, phenolic acids and amino acids, and enzymes. Among them, polyphenols, alkaloids, and terpenoids are some of the most reported natural products with autophagic regulation.

1. Polyphenols

Polyphenols are widely distributed in the plant realm and are a large class of important natural compounds. Polyphenols have a flavonoid structure and are highly resistant to the abundance of reducing chemical groups such as phenolic hydroxyl groups, which contribute to its oxidizing properties. The diverse structure of polyphenolic compounds leads to that it is easy to interact with a variety of proteins such as kinases and receptors. In addition, polyphenols are also the most widely reported natural products which are most closely related to autophagy regulation.

2. Alkaloids

Alkaloid is mostly found in plants, so it is also called plant alkali. It is a kind of organic basic compound containing nitrogen and special complex cyclic structure, in which nitrogen is mostly contained. That is most of the molecules contain nitrogen-containing heterocyclic rings. Pyridine, hydrazine, quinoline, anthracene, etc., and a few are amine compounds. A large number of studies have involved the interaction between alkaloids and autophagy.

3. Terpenoid

As a sort of widely existing compound, terpenoid refers to a derivative having the formula $(C5H8)_n$ and its oxygen content and different degree of saturation, which can be regarded as a kind of natural compound which is formed by isoprene or isopentane in various ways. Furanodiene is a natural product of terpenoid extracted from Chinese herbal medicine. The various active ingredients in the sputum have a pro-apoptotic and proliferative effect on a variety of tumor cell lines, such as furandiene promotes apoptosis of lung cancer 95-D cells and induces cell cycle arrest in G1 phase. With furandiene treatment, LC3-II protein expression was significantly increased in 95-D cells, thereby inducing the occurrence of autophagy in tumor cells.

Since natural products can be converted by chemical reaction or in vivo metabolism under certain conditions, to study the in vivo regulation of natural products on autophagy is more important, and it is necessary to clarify the metabolites and metabolic kinetics of these natural compounds on their regulation to the autophagy process.

Chapter 53
Natural Product Regulates Autophagy in Cancer

Yilixiati Xiaokaiti and Xuejun Li

Abstract Anti-cancer effect of natural products has been widely known. As a sort of multi-target anti-cancer agents, natural compound's regulation on autophagy in cancer cells has been studied as a promising research to reveal the mechanism in oncogenesis, as well as a potential short way to anti-cancer drug discovery. In this chapter, we reviewed the cancer-autophagic-related studies on several natural product compounds. It was concluded that natural product compounds directly or indirectly regulated most of the target proteins on the autophagic signal pathways. Considering we have not seen the whole clear atlas of autophagy in oncogenesis yet, it is hard to raise up any conclusion that autophagy is always playing a positive role in oncogenesis and cancer progression.

Keywords Natural product · Autophagy · Cancer · Oncogenesis · Multi-target

53.1 Inroduction

The role of autophagy in tumorigenesis and development has been elaborated in the previous chapters of this book, and the duality of autophagy on tumor regulation remains a controversial subject for further study. Tumorigenesis may be prevented through autophagic regulation, but once tumorigenesis happened, an increase in autophagy flux tends to promote tumor cell survival and growth (Levy et al. 2017).

There is evidence that autophagy blocked the conversion of normal cells to cancer cells, autophagy cleared the accumulation of damaged organelles or proteins, and further activated programmed cell death when cells were severely damaged, which also provides checkpoint to avoid tumorigenesis. In addition, autophagy inhibits tumorigenesis by maintaining chromosome stability, inhibiting inflammation and angiogenesis, and promoting oncoprotein degradation (Zhan et al. 2018). However, in the absence of nutrients, hypoxia, metabolic stress, and treatment-induced cellular stress or drug resistance, autophagy may promote the survival of established

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tumor cells and produce resistance to radiation, chemotherapy, and targeted drug therapy (Rebecca and Amaravadi 2016). If autophagy is over-activated, it may lead to caspase-independent non-apoptotic cell death. Autophagy under certain special conditions may cause apoptosis, such as dramatic changes in Bcl-2 family protein levels. In recent years, immunotherapy has attracted widespread attention in the treatment of tumors. However, autophagy also plays a double-edged role in immunotherapy. The exact role and mechanism of autophagy in cancer immunotherapy is still unclear.

It should be mentioned that cisplatin, alkaloids, antimetabolic anti-cancer drugs, tyrosine kinase inhibitors, and other targeted therapeutic drugs, as well as radiotherapy, induce autophagy in cancer cells. However, the relationship between the effects of these treatments and the occurrence of autophagy still requires extensive research. A clinical trial with more than 30 samples investigated the anti-cancer effects of autophagy modulators in combination with cytotoxic chemotherapeutic drugs or targeted drugs in a variety of cancer cases. For example, chloroquine and hydroxylated chloroquine, which are commonly used in the treatment of malaria and rheumatoid arthritis, inhibit autophagosome acidification, therefore inhibiting autophagy. In anti-cancer therapy, chloroquine was combined with cisplatin or PI3K inhibitor LY294002 or mTOR inhibitor rapamycin, and the results showed that there is no significantly increased sensitivity to the anti-cancer treatment or autophagy inhibitors used above. Knockdown of Atg12, Beclin 1, or the use of bottromycin failed to simulate the above results. Clinical trial results provide important evidence for our in-depth understanding of autophagy and its role in tumor physiology and provide fresh ideas for the development of adjuvant anti-cancer therapy targeting autophagy.

It has been shown that natural products affect autophagy by regulating ROS levels in tumor cells, as well as directly regulate autophagy (induced autophagy or autophagy inhibition) (Table 53.1).

53.2 Polyphenol

A large number of studies have reported that polyphenols showed inhibitory effects on tumors alone or in combination with other anti-cancer therapy, and the mechanisms involve the regulation of autophagy. Such as catechins, resveratrol, quercetin, and curcumin, these polyphenols prevent the tumorigenesis by inducing autophagy, and may also contribute to its anti-aging effect. In addition to the induction of autophagy, it has been reported that some polyphenols have an inhibitory effect on autophagy, which has certain benefits for the treatment of cancer, especially for radiotherapy, chemotherapy, and targeted drugs. The latest research found that polyphenols can be used as an adjunct to the development of cardiotoxicity induced by doxorubicin, a chemotherapeutic drug (Shabalala et al. 2017). In the animal experiments and clinical researches, the study of polyphenolic natural products as adjuvant therapy in chemo or targeted therapy proves that regulating autophagy is a key mechanism for

Table 53.1 Natural product regulated cancer cell lines (or tumor tissue), autophagy-related mechanisms, and possible targets

Compound name	Cell line and tissue	Mechanism and target protein
Curcumin	Brain Bladder Prostate Colon Brain Mesothelioma Colon Endothelial Colon	Akt/mTOR/p70S6 K ERK1/2 CML Bcl-2 Akt Bcl-xL ROS PI3K/Akt/mTOR Beclin 1 and p62/SQSTM PI3K/Akt/mTOR and FOXO1 TFEB/Lysosome
EGCG (Epigallocatechin-3-gallate)	Hep3B Macrophage-like cell line Raw264.7 Mesothelioma cells BAEC HepG2 4T1 CAR	Atg5 Beclin 1 NOS LC3 ROS ROS PKC-β LC3 II LC3-I, LC3 II Beclin 1, ATG5, LC3B Atg5, Atg7, Atg12, Beclin 1, and LC3B-II AKT/STAT3
Resveratrol	Ovarian Salivary gland Ovarian Lung Colorectal Breast Cervical Gastric Brain Fibroblast, Cervical CML Lung Hepatoma Liver Colon Brain Cervical Osteosarcoma Melanoma Cervical, breast Lung	PELP1/HRS Akt/mTOR/p70S6K PELP1/HRS PI3K/Beclin 1/Lamp2b Akt/PKB/mTOR/p70S6K Cathepsin L Dihydroceramide desaturase Beclin 1 p70S6K JNK/p62, AMPK/mTOR SIRT1/PARP-1 SIRT1, AMPK, HIF-1α SIRT1 ATAD3A WIPI-1 Inhibiting autophagy

(continued)

Table 53.1 (continued)

Compound name	Cell line and tissue	Mechanism and target protein
Quercetin	Gastric carcinoma Colon Fibroblast-breast Gastric Rat mesothelial Breast, Cervical Ovarian Breast	Akt-mTOR and HIF-1α Ras ROS Akt/mTOR and HIF-1 HSP72/jnk and Beclin 1 mTOR/eIF4E-BP1/p70S6K Akt-mTOR and glycolysis
Genistein	Rat hepatocytes Ovarian Lung A549	Cytokeratin Akt PDE4A4vand p62/SQSTM1 N-CoR/Hsc70 Autophagic flux
Rottlerin	Prostatic carcinoma Fibrosarcoma Breast cancer SGC7901 and MGC803	PKCδ/TG2 pathway NF-κB PKCδ/TG2 independent pathway mTORC1 Rapamycin kinase and Skp-2
Berberine	HepG2 and MHCC97-L NCI-H2452	Atg5 Akt P38/MAPK Beclin 1 mTOR LC3 - II, p62, inhibiting autophagy
Matrine	HepG2 and SGC-7901	Pancreatic cancer Beclin 1 STAT3

natural products to overcome anti-cancer drug resistance and enhance the therapeutic effect of chemotherapy drugs. But more and more extensive and in-depth research is needed.

53.2.1 Curcumin

As a type of polyphenol compound extracted from *Curcuma longa,* Curcumin's regulation on autophagy is involved in the PI3K/Akt/mTOR signaling pathway and NF-κB-regulated proteins. A number of studies have confirmed that curcumin induces G2/M arrest and autophagy by inhibiting the activation of Akt/mTOR/p70S6K and ERK1/2 signaling pathways in cancer cells. Shinojima et al. observed that curcumin inhibited solid tumor proliferation mainly through autophagy rather than NF-κB (Shinojima et al. 2007).

Studies have shown that curcumin reduced the expression of Sp protein, and the overexpression of this protein in gastric cancer and pancreatic cancer is closely related to tumor invasion and poor prognosis, downregulation of EGFR (Sp protein regulatory gene, autophagy inhibition) expression, inhibition of Akt phosphorylation, induction of increased LC3 expression, and death of bladder cancer cells. Mosieniak et al. (2012) demonstrated that the senescence of colon cancer cell lines is accompanied by the development of autophagy with upregulated expression of Beclin 1 and p62/SQSTM1 proteins. Inhibition of autophagy by Atg5 siRNA interference reduces curcumin-induced cellular senescence but does not increase cell death. This study reveals that curcumin-induced cellular senescence is associated with autophagy, and its specific mechanisms require more deep research.

It has been reported that curcumin induced autophagy and apoptosis by downregulating Bcl-2 protein in chronic myeloid leukemia cells. The reverse of these effects by treating cells with autophagy/lysosomal inhibitor bafilomycin or caspase inhibitor zVAD-FMK, respectively, confirmed curcumin's autophagy-mediated inhibitory effect on chronic myeloid leukemia cells (Jia et al. 2009). In prostate cancer cells, curcumin induced autophagy and promoted cell death by downregulating Bcl-2 protein family member Bcl-xL. Curcumin does not induce cleavage of procaspase-8, −9, −3, and −7 or PARP, but results in the formation of LC3B-II isoforms and an increase in autophagosomes.

Curcumin treatment reversed the LC3-I/LC3-II ratio and promoted the breakdown of SQSTM1 and therefore induced the formation of autophagosomes in human colon cancer cells. Curcumin-induced autophagy can be blocked by the antioxidant NAC, suggesting that curcumin may act by promoting ROS production, autophagosome formation, and autolysosomal cleavage. Batroxomycin-induced SQSTM1 protein degradation further confirms that activation of autophagy may lead to cell death. Kim et al. reported that the anti-tumor effect of curcumin on oral squamous cell tumor involved the ROS production and presented anti-tumor activity through apoptosis and autophagy.

In addition, for glioblastoma, curcumin also induced autophagy in vitro and in vivo. Curcumin is less toxic to normal cells, especially in glial cells (GICs), the mechanism of action is through regulation of ERK1/2 signaling pathway (Zhuang et al. 2012).

Several studies have shown that curcumin regulated autophagosome and autolysosome formation, which enhanced the autophagic flux of human colon cancer HCT116 cells and mouse embryonic fibroblasts (MEFs), and then promoted lysosomal function. Curcumin-mediated lysosomal activation is mediated by mTOR inhibition and increased lysosomal acidification and enzymatic activity. Curcumin treatment activated several essential nuclear transcription factors that regulate autophagy and lysosomal and transcript factor EB (TFEB). Curcumin directly binds to TFEB, promotes nuclear translocation of TFEB, or increases the transcriptional activity of TFEB (Zhang et al. 2016). It has also been reported that a curcumin derivative (IHCH) inhibits the growth of A549 cells and induces the formation of autolysosomes in a dose- and time-dependent manner.

Curcumin has been shown to induce not only tumor cell apoptosis, but also synergistic effects with various FDA-approved drugs. However, the main reasons that prevent curcumin from becoming an anti-tumor drug are its low bioavailability, its low absorption rate, and poor in vivo distribution and biological metabolism. In order to solve the above problems, it is necessary to improve the absorption and bioavailability of curcumin by means of formulation modification, by using the nanomaterials, micelles, and phospholipid complex packaging. It should be emphasized that curcumin produces a variety of metabolites in the body, including glycosylation products, sulfation products, tetrahydro-, hexahydro-, and decahydrocurcumin, and all metabolites exhibit anti-tumor activity.

53.2.2 Epigallocatechin-3-Gallate (EGCG)

EGCG is a type of polyphenol extracted from green tea. Studies have shown that low concentrations of EGCG (10 μM) induced macrophage and tumor cell autophagy to degrade endotoxin-induced high mobility group B-1 (HMGB1) aggregation, resulting in anti-inflammatory effects. In contrast, high concentrations of EGCG (100 μM) inhibit autophagy, leading to apoptosis and anti-tumor effects. In addition, the combination of EGCG with certain anti-tumor drugs can also produce synergistic effects and inhibit autophagy.

As a biomarker of hepatocellular carcinoma (HCC), high level of α-fetal-associated protein (AFP) suggests malignant tumor differentiation and poor prognosis. Studies have found that EGCG effectively reduced AFP secretion in human hepatoma HepG2 cells and promoted autophagy-induced degradation of AFP aggregates in HepG2 cells. In addition, large-scale all-atom molecular dynamics simulation revealed a new molecular mechanism of EGCG. In addition, it was found that EGCG directly interacts with LC3-I protein and exposes the key site Gly-120 of LC3-I to other important binding partners, such as 1,2-divinyl-sn-glycerol-3-phosphoethanolamine, promoting the synthesis of autophagosome-labeled LC3-II, which provided a potential molecular basis for the prevention and treatment of hepatocellular carcinoma (Zhao et al. 2017).

It was also found that EGCG can sensitize the efficacy of several chemotherapeutic drug doxorubicin (DOX) to enhance its therapeutic effect on hepatocellular carcinoma. Electron microscopy and fluorescence microscopy confirmed that DOX significantly increased autophagic vesicles in hepatoma Hep3B cells. Results of immunoblotting and trypan blue assays showed that DOX increased the autophagic flow of about 45% of dead cells. In contrast, quantitative RT-PCR and immunoblotting showed that EGCG dose dependently inhibited autophagy signals, and 40 μg/ml EGCG treatment decreased the expression of Atg5 and beclin 1. In addition, EGCG treatment significantly enhanced the role of DOX inhibition of tumor cell growth. Combination therapy increases cell death by approximately 40–60% and synergistically enhances apoptosis, antagonizing DOX-induced autophagy. As a kind of autophagy inhibitor, rapamycin significantly inhibited the anti-cancer effect of DOX

or a combination of EGCG. On the other hand, the use of small interfering RNA targeting chloroquine-related autophagy genes Atg5 and beclin 1 inhibited autophagy, resulting in a significant increase in liver cancer cell death. In the subcutaneous transplantation of the Hep3B cell tumor model, the combination treatment of DOX and EGCG inhibited tumor growth by about 25% and apoptotic cells by 50% compared with DOX treatment. In addition, immunohistochemistry analysis indicated that the suppressed tendency of autophagic hallmark microtubule-associated protein LC3 expressions was consistent with thus combined usage in vitro (Chen et al. 2014).

Malignant mesothelioma is an asbestos-related fatal disease and there is currently no effective treatment. It was found that EGCG induced apoptosis in five sorts of human mesothelioma cell lines in a dose-dependent manner, which was related to EGCG-induced increase in reactive oxygen species (ROS) and damage to mitochondrial membrane potential. It was also found that EGCG induced autophagy, but when autophagy was inhibited by chloroquine, EGCG-induced cell death was enhanced (Satoh et al. 2013). These results indicate that the inhibitory effect of EGCG on mesothelioma is related to its induction of apoptosis and autophagy.

Recent studies have shown that EGCG induced autophagy in breast cancer 4T1 cells by regulating the expression levels of autophagy-related proteins beclin 1, ATG5, and LC3B in a concentration-dependent manner. The research on the molecular mechanism of EGCG on drug-resistant oral squamous cell carcinoma illustrated that EGCG inhibited cisplatin-resistant oral cancer CAR cell line and significantly increased Bax, cleaved caspase-9, cleaved caspase-3, Atg5, Atg7, Atg12. Expression of proteins such as beclin 1 and LC3B-II significantly decreased the expression of Bcl-2, phosphorylated AKT (Ser473), and STAT3 (Tyr705) in CAR cells. Importantly, EGCG showed a dose-dependent inhibition of protein and gene expression of multidrug resistance 1 (MDR1). It is clear that downregulation of MDR1 levels and changes in AKT/STAT3 signaling pathway promoted EGCG-induced apoptosis and autophagy in CAR cells. It suggests that EGCG has the potential to treat oral cancer and may play a role in the prevention of long-term oral cancer (Yuan et al. 2017).

53.2.3 Rottlerin

Rottlerin (5, 7-dihydroxy-2, 2-dimethyl-6-(2, 4, 6-trihydroxy-3-methyl-5-acetylbenzyl)-8-cinnamoyl-1, 2-chromine), also known as crude purotoxin, is extracted from Philippine bitter tea. Rottlerin induces autophagy through multiple pathways such as PKCδ/TG2 pathway, PKCδ-independent pathway, and mTORC1 pathway in prostate cancer, fibrosarcoma, breast cancer, gastric cancer, bladder cancer, and other types of cancers.

In the breast cancer and colon cancer cell models, rottlerin has antioxidant activity and inhibits NF-κB (Maioli et al. 2009). Upregulation of PKCδ and TG2 levels led to NF-κB activation, and inhibition of this pathway results in autophagy and cell death. Rottlerin has been recognized as the inhibitor of PKCδ. Rottlerin induced excessive autophagy by PKCδ and TG2 leading to prostate cancer cell death. Recent

studies have demonstrated that rottlerin induced apoptosis in fibrosarcoma cells via a PKCδ-independent pathway.

In breast cancer cell lines under normal nutrient deficiencies, rottlerin induced autophagosomes aggregation by blocking the mTORC1 pathway (Balgi et al. 2009). Rottlerin induced AMPK activation, reduced intracellular ATP levels, induced autophagy in tumor cells, or activated cyclin-dependent kinase (CDK) inhibitory protein p27 through AMPK and SIRT1/FOXO pathways, as a sequence, promoted autophagy. Rottlerin-induced autophagy may involve a number of signaling pathways and many mechanisms that induce autophagy, thereby causing cell death. However, the decisive role is still the external environment, the critical state of cells that trigger or inhibit apoptosis, and activation and inhibition of related signaling pathways. Pharmacokinetic results in a mouse-transplanted solid tumor model showed that tumor tissue has a good absorption effect on rottlerin, so rottlerin or its derivatives have the potential to be developed to induce autophagy and lead to the promising drugs.

Studies have shown that rottlerin induced autophagy and apoptosis of SGC-7901 and MGC-803 gastric cancer cell lines and inhibited cell migration and invasion. Moreover, rottlerin increased the expression of LC3β and enriched autophagosomes, while the expression levels of rapamycin kinase and S phase kinase-associated protein 2 (Skp-2) associated with autophagy were downregulated (Song et al. 2018). It is suggested that rottlerin may inhibit the invasion of gastric cancer cells and promote the apoptosis of gastric cancer cells, which may be mediated by autophagy activity.

In addition, it was found that rottlerin significantly increased apoptosis by inducing autophagy, inhibiting the viability of EJ human bladder cancer cells in a dose- and time-dependent manner. Rottlerin treatment induced autophagy, which was characterized by increased expression of LC3-II and increased autophagosomes. Elevated levels of LC3-II and autophagosomes suggest that autophagy may contribute to apoptosis in these cells (Qi et al. 2016).

53.2.4 Genistein

Genistein (4, 5, 7-trihydroxyisoflavone) is an isoflavone natural product extracted from legumes and has anti-tumor activity. Genistein induced cell death through apoptosis and autophagy pathways, and also reversed tumor chemoresistance by altering the role of apoptotic signals.

Genistein protected the cytokeratin network in stress, nutrient deficiencies, and growth factor deficiency. A number of studies have shown that genistein overcomes the okadaic (a potent inhibitor of autophagy)-induced damage of mouse liver cells through cytoskeleton and keratin recombination, suggesting that keratin filaments may be involved in autophagy. Genistein has a two-way cytotoxic effect on promoting apoptosis and autophagy in cervical cancer, inhibiting the glucose absorption and oxidative phosphorylation substrates and fatty acid synthesis substrate (methyl pyruvate), thereby effectively eliminating promoting autophagy of genistein.

Christian et al. (2010) reported that genistein inhibited autophagy in cervical cancer cells by inhibiting PDE4A4 aggregation. PDE4A4 interacts neither with autophagosomes nor with aggregates, but with p62 (SQSTM1) protein. Due to p62 and LC3, the interaction promoted the autophagosome formation, thus inhibiting the aggregation of PDE4A4 which may induce the development of autophagy.

Although genistein induces autophagy and apoptosis in a variety of tumor cells, pharmacokinetic or ADME studies have revealed that genistein has low oral bioavailability due to metabolic enzymes and transporters. Therefore, in the apoptosis-resistant cancer, the action efficiency of genistein still needs to be improved.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a transmembrane cytokine that selectively induces apoptosis in a variety of tumor cells and is a promising tumor suppressor gene (Nazim and Park 2015). Inhibition of autophagy flux has increasingly been recognized as a good and novel cancer therapy. Genistein induced TRAIL-mediated apoptosis in human adenocarcinoma A549 cells through TRAIL signal pathway. Notably, genistein treatment significantly increased LC3-II and p62 protein levels. The combination of genistein and TRAIL treatment increased the accumulation of LC3-II, p62, activated caspase-3, and activated caspase-8, inhibiting autophagy flux, indicating that genistein enhances drug-resistant A549 by inhibiting autophagy flux, as well as TRAIL-induced tumor cell death in adenocarcinoma cells.

53.2.5 Quercetin

Quercetin (3, 3′, 4′, 5, 7-pentahydroxyflavone) is a type of flavonoids natural product and is abundant in fruits, vegetables, plant stems, and leaves; it interacts with a variety of molecular, organelle, and tumor development related pathways, and therefore presents anti-tumor activity.

In order to confirm the effect of quercetin on autophagy, Psahoulia et al. (2007) used 3-MA to act on RAS gene-modified colon cells, inhibiting the formation of vacuoles, whereas the caspase inhibitor zVAD-FMK failed to inhibit quercetin-induced vacuolar formation, and the above results confirmed that quercetin induced autophagy, which was caspase-independent.

For gastric cancer cells, quercetin induced autophagy through activating several hub knots in autophagy, but after using the autophagy inhibitor chloroquine or knockout of Atg5 or beclin 1 gene, apoptosis of gastric cancer cells is significantly enhanced, suggesting quercetin-induced autophagy protected tumor cells against apoptosis. Further studies have shown that quercetin activates autophagy by modulating Akt-mTOR and HIF-1α signaling pathway (Wang et al. 2011). The above studies on tumor cells and xenograft tumor animal models have demonstrated that quercetin simultaneously induces autophagy and apoptosis.

In addition, quercetin also promotes the removal of damaged mitochondria by autophagy/mitochondrial autophagy in oxidative stress. Therefore, it can be

considered that fibroblasts around cancer cells provide nutrition and energy for mitochondrial production of adjacent cancer cells by reversing the Warburg effect.

Quercetin induces the intracellular vesicle and autophagosome formation by upregulating autophagy-associated marker proteins in epithelial cancer cells, which form cell cycle arrest and induce apoptosis. Prior to the formation of autophagosomes, mTOR activity was detected to be inhibited, accompanied by a significant decrease in phosphorylated substrate levels, including the endoplasmic reticulum S6 subunit (phosphorylation by p70S6 kinase) and elF4 (via inhibiting phosphorylation of elF4 inhibitory protein 4E-BP1). Quercetin also induces excessive autophagy by inhibiting proteasome activity and mTOR activity, leading to cancer cell death. Therefore, quercetin has strong anti-tumor activity, not only through cell cycle arrest and apoptosis, but also through regulating key autophagy signaling pathways such as Akt-mTOR and HIF-1α.

Studies on the bioavailability and metabolic kinetics of quercetin in rats showed that after 53% of quercetin was administered by gavage, 93.8% of quercetin was present in the structures of sulfonylation and glycosylation in the blood circulation. The original structure of quercetin was not detected in the blood.

Tumor metastasis is one of the main causes of death in cancer patients. Inhibition of tumor metastasis by inhibiting glycolysis (the main pathway of tumor cell energy supply) is one of the popular research fields in cancer therapy. Studies have found that quercetin inhibits the breast cancer metastasis by inhibiting the Akt-mTOR pathway, inducing autophagy, and by inhibiting glycolysis. Quercetin inhibits glucose uptake and lactic acid production, and successfully blocks cell glycolysis and reduces glycolysis-associated proteins pyruvate kinase M2 (PKM2), glucose transporter 1 (GLUT1), and lactate dehydrogenase A (LDHA). It is suggested that quercetin may inhibit glycolysis by reducing the acidity of the tumor microenvironment. The application of autophagy inhibitor 3-MA and Akt-mTOR pathway inducer IGF-1 further demonstrated that quercetin inhibited cell migration and glycolysis by autophagy mediated by Akt-mTOR pathway. In vivo studies have shown that quercetin treatment inhibits tumor growth and metastasis by inhibiting p-AKT/AKT, which in turn induces autophagy, thus inhibiting glycolysis (Jia et al. 2018). This study found for the first time that quercetin inhibits cell migration and glycolysis through autophagy induced by Akt-mTOR pathway, thereby inhibiting the breast cancer oncogenesis and providing a potential therapeutic target for breast cancer treatment.

53.2.6 Resveratrol

Resveratrol (3,5,4-trihydroxystilbene) is a phytoalexin present in grapes, nuts, and red wine, which has chemopreventive and multi-target properties, and its targets depend on types of cell lines and environmental conditions. Opipari et al. (2004) confirmed that resveratrol induces cell death by promoting autophagy in five types of cervical cancer cell lines, suggesting that resveratrol may have a lethal effect on apoptosis-resistant tumors, and resveratrol initiates nutrient deficiency. The response

signaling pathway, for example, reduces the level of phosphorylated Akt in cervical cancer cells and the expression of mTOR. Studies have demonstrated that in chronic myeloid leukemia cells, resveratrol initiates autophagy and leads to cell death by activating JNK-mediated overexpression of p62/SQSTM1 and AMPK/mTOR signaling pathway activation machinery. Resveratrol also upregulates the expression of several tubulin subunits, which play an important role in the movement of autophagosomes.

It has been reported that resveratrol is not cytotoxic to human colorectal cancer LDL1 cells in acute short-term treatment (for 2 h), whereas repeated and prolonged (48 h) resveratrol exposure initiates autophagy-dependent cytotoxicity. Inactivation of PI3K/Beclin 1 and Lamp2b can significantly reduce the cytotoxicity of resveratrol (Trincheri et al. 2008). After gene silencing of Lamp2b, fusion of autophagosomes with lysosomes was abolished. In addition, studies using this model also found that the caspase inhibitor zVAD-FMK inhibited cell death but did not inhibit autophagy. This study shows us two new ways of producing cytotoxic effects of resveratrol, in which autophagy has a two-sided effect. Initial autophagy initiates a response to stress signaling and, in the later stages, responds to the mechanism of caspase-dependent apoptosis. In another study, resveratrol upregulated the ROS level by inducing caspase-8 and caspase-3 splicing and upregulating LC3-II expression in colon cancer cells. This effect was blocked by NAC (N-acetyl cysteine).

An inhibitory protein of endogenous cathepsin L, SCCA1, is widely expressed in uterus and cervical cells. Hsu et al. (2009) found that the lysosomal pathway of cathepsin L-SCCA1 and autophagy mediates the toxic effects of resveratrol on uterine cells. In this cell model, autophagy inhibitors wortmannin or asparagine was used to reduce resveratrol-induced cell death. In glioma cells, resveratrol-induced autophagy can inhibit resveratrol-induced apoptosis. Autophagy plays a different role in apoptosis, which leads to the death of glioma cells, whereas autophagy delays the apoptotic process and protects cell survival. It can be seen that autophagy inhibitors may have the potential to enhance the anti-tumor activity of resveratrol.

SIRT1 is one of the most popular targets for resveratrol, which activates SIRT1 and induces the development of autophagy and apoptosis (Wang et al. 2018a, b). However, Armour et al. found that resveratrol inhibits autophagy in response to nutrient deficiencies in this cell line by a SIRT1-independent pathway in some tumor cell lines. Resveratrol induces autophagy by regulating SIRT1 and PARP in lung cancer cell lines mediated by tobacco-mediated oxidative stress. Resveratrol decreased ATP concentration and upregulated SIRT1 expression in liver tissues of wild-type mice with endotoxin intervention, and also increased HIF-1α expression and promotes autophagy, whereas in SIRT1 knockout mouse model, the above effects cannot be observed.

However, it has recently been reported that resveratrol induced protective autophagy in non-small cell lung cancer by activating SIRT1, inhibiting Akt/mTOR, and activating p38-MAPK. They found that the combination of the autophagy inhibitor 3-MA or the SIRT1 inhibitor nicotinamide significantly inhibited proliferation and promoted apoptosis compared with the resveratrol 200 μM group alone. It indicated that resveratrol-induced autophagy may promote the survival of NSCLC

cells as a protective mechanism, while inhibition of autophagy may enhance the anti-tumor effect of resveratrol. Furthermore, resveratrol treatment inhibited Akt/mTOR, while p38-MAPK was activated in NSCLC cells in a dose-dependent manner. The combination of IGF-1 to activate the mTOR pathway or inhibit the p38-MAPK pathway with doramapimod significantly inhibited cell proliferation and increased apoptosis of non-small cell lung cancer cells compared to 200 μM resveratrol alone (Wang et al. 2018a, b). These studies indicate that resveratrol-induced autophagy may be a protective mechanism that promotes survival of NSCLC cells, thus inhibiting autophagy enhances the anti-tumor activity of resveratrol in non-small cell lung cancer.

Resveratrol is metabolized in the body to a glycosylated, sulfated form. Oral resveratrol bioavailability tends to zero due to its excessive metabolic rate (Wenzel and Somoza 2005). However, resveratrol induces autophagy in a variety of tumor cells, and its multi-target anti-tumor activity has led drug developers to attach great importance to structural modification and drug development.

53.3 Alkaloids

Alkaloids are rich treasure trove of natural products. Current research shows that alkaloids exert obvious anti-proliferative and metastatic effects on tumors. Among them, camptothecin and vinblastine have been successfully developed into anti-tumor drugs. It has also been reported that cyclovirobuxine D (CVB-D) has a dose- and time-dependent induction effect on autophagy in breast cancer cell lines. Berberine, evodia, corni, matrine, piperine, phloretin, and tetrandrine are other alkaloids under investigation.

53.3.1 Berberine

Berberine is an isoquinoline alkaloid, which has a wide range of biological activities such as anti-inflammatory, antibacterial, anti-diabetic, anti-ulcer, sedative, protecting against myocardial ischemia–reperfusion injury, dilates blood vessels, inhibiting platelet aggregation, and protecting liver and nerves. They have been used for the treatment of diarrhea, neurasthenia, arrhythmia, diabetes, and so on.

The in vitro and in vivo studies have shown that berberine has strong anti-tumor properties through a multifaceted mechanism that interferes with tumor progression. In addition, berberine was also found to induce apoptosis and autophagy in hepatoma cells HepG2 and MHCC97-L (Wang et al. 2010). In the presence of 3-MA or the interfering autophagy gene Atg5, berberine-induced hepatoma cell death is reduced. Further studies have found that berberine may induce apoptosis by increasing Bax expression and may also inhibit Akt activity and upregulation of P38/MAPK signaling, which in turn activates beclin 1, inhibits mTOR signaling, and induces autophagic

HepG2 and MHCC97-L cell death. Compared with clinically used anti-cancer drugs, its cytotoxicity is weak, but it inhibits invasion and metastasis as well as tumor angiogenesis. Combined with chemotherapy drugs or radiotherapy, berberine improved the treatment effect.

However, a study has shown that berberine induces mitochondria-mediated apoptosis in human malignant pleural mesothelioma NCI-H2452 cells but produces protective autophagy (Yao et al. 2018). This study found that berberine inhibited the proliferation of NCI-H2452 cells in a dose- and time-dependent manner and may induce apoptosis through a caspase-9-dependent mitochondrial pathway. In addition, berberine induced autophagy, showing accumulation of LC3-II and decreased expression of p62. Further use of apoptosis inhibitors and autophagy inhibitors, or autophagy inducers, found that apoptosis is the main pathway for berberine-induced cell death in NCI-H2452 cells. However, berberine-induced autophagy may be an adaptive response to anti-tumor drugs and has a protective effect on malignant pleural mesothelioma cells. Inhibition of autophagy enhances berberine-induced apoptosis. Therefore, inhibition of autophagy may be an effective strategy for the treatment of malignant pleural mesothelioma.

53.3.2 Evodiamine

Evodiamine is a quinolone alkaloid and is the main active compound of Chinese herbal medicine *Evodia*. It has anti-anxiety, analgesic, anti-inflammatory, anti-allergic, protective myocardial ischemia–reperfusion injury, vasodilation, and anti-tumor effects. The study found that Chinese herb medicine *Wujing* activated autophagy in tumor cells, which mainly showed protective effects on tumor cells. It is currently believed that redox of cells is extremely important for controlling apoptosis or autophagy. Evodia induces the production of ROS and NO in HeLa cells in a time-dependent manner, while Evodia also induces autophagy, but pretreatment with 3-MA, a specific inhibitor of autophagy, dose dependently reduces cell viability, indicating autophagy played a protective role in cancer cell survival. These findings may help to elucidate the regulation of autophagy and apoptosis in the redox state of cells and their effects in anti-tumor therapy (Yang et al. 2008).

Glioblastoma is one of the most aggressive types of brain tumors. The median survival rate for patients with glioblastoma (World Class IV) was < 15 months. WZY-321 is a novel evodiamine derivative. Studies have shown that WZY-321 inhibits the proliferation of SHG-44 cells in a dose- and time-dependent manner by promoting apoptosis and inducing cell cycle arrest. IL-3α and beclin 1 levels induce autophagy to produce an effect (Sun et al. 2019).

53.3.3 Matrine

Matrine is extracted from the plant *Sophora flavescens* and has a wide range of pharmacological activities. It has been used to treat bacterial dysentery, enteritis, arrhythmia, and anti-tumor. Although matrine inhibits the proliferation of cancer cells at a relatively high concentration (milli-mole), it has no significant effect on the survival rate of normal cells. Matrine can cause apoptosis and autophagy in cancer cells, such as liver cancer HepG2 cells and SGC-7901 gastric cancer cells. Studies have shown that matrine dose and time dependently inhibits the proliferation of HepG2 hepatoma cells and induces cell cycle arrest and HepG2 cell apoptosis in G1 phase. Electron microscopy studies showed that HepG2 cells formed abundant autophagic vacuoles after matrine treatment and showed more MDC-labeled particles. After the autophagy inhibitor 3-MA, the number of autophagic vacuoles was greatly reduced. The above results indicate that matrine induces autophagy and apoptosis in HepG2 cells. Real-time quantitative RT-PCR results showed that the expression levels of Bax and Beclin 1 increased, suggesting that Beclin 1 may be involved in matrine-induced autophagy, and the pro-apoptotic mechanism of matrine may be related to the upregulation of Bax gene expression (Zhang et al. 2010).

Recently, it has been reported that matrine significantly reduced the pancreatic cancer proliferation by reducing mitochondrial metabolism and energy levels in vitro and in vivo. Supplementation with pyruvate or α-ketoglutarate significantly reversed the growth of pancreatic cancer cells inhibited by matrine. The mechanism of action may be that matrine downregulates STAT3, inhibits autophagy, weakens the function of lysosomal proteases, and inhibits mitochondrial energy production. In addition, studies have shown that the inhibition of matrine on the growth of pancreatic cancer depends on the mutation of KRAS oncogene. Taken together, this study demonstrates that matrine can inhibit the growth of KRAS mutant pancreatic cancer by inhibiting autophagy-mediated energy metabolism (Cho et al. 2018).

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Chapter 54
Regulation of Autophagy in Neurodegenerative Diseases by Natural Products

Shuaishuai Liu and Xuejun Li

Abstract Neurodegenerative diseases mainly include Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD). It is now found that these diseases may be related to autophagic dysfunction. The mechanism is due to abnormalities in autophagy, which lead to abnormal or misfolded proteins accumulating in the cytoplasm, nucleus, and extracellular inclusion bodies, causing neuronal organelle damage and synaptic dysfunction. Since these diseases are much complex, the effect of monotherapy is not significantly affected. There is still a need to strengthen the study of anti-neurodegenerative drugs. Natural products should be a good source for the new drug discovery since most of natural products are multiple-target compounds. In this chapter, we reviewed some progress on studying resveratrol, curcumin, tripterine, and paeoniflorin. These natural products can eliminate abnormal protein aggregates by regulating autophagy, and thereby these compounds are promising to be used in prevention and treatment of neurodegenerative diseases in the future.

Keywords Natural product · Autophagy · Neurodegenerative diseases · Multi-target

54.1 Introduction

Neurodegenerative diseases include Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and so on. In recent years, many studies have found that the development of these diseases may be related to autophagic dysfunction. Abnormal autophagy leads to abnormal or misfolded proteins accumulating in the cytoplasm, nucleus, and extracellular inclusion bodies, and causes neuronal organelle damage and synaptic dysfunction, leading to the development of neurodegenerative diseases (Nakamura et al. 2018; Zhang et al. 2017).

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Neurons rely on higher basal autophagy to survive than non-neuronal cells. Defects in autophagy significantly damage the neurons at the end of the division, and the loss of neurons can lead to neurodegenerative diseases. Aging mitochondria are one of the sources of ROS that activate proinflammatory cytokines and cause inflammation. Mitochondria play an important role in programmed cell death, increasing mitochondrial membrane permeability induces apoptosis, and degradation of damaged mitochondria by autophagy protects cells from lethal damage and inhibits proinflammatory ROS production. However, not only inhibition of mitochondrial degradation can damage cells, but also inhibition of degradation of protein aggregates can also lead to cell damage. It is because that the defect autophagy inhibits the degradation of Huntingtin, amyloidogenic Aβ, and α-synuclein and causes HD, AD and PD. In hereditary Huntington's disease, there are increased polyglutamine bundles and misfolded prion proteins, which hinder their degradation (Nabavi et al. 2018). It is now found that most of PD and AD are not caused by heredity, and the main reason may be the aging process (Pallauf and Rimbach 2013).

In the AD, the autophagy process is slowed down or even interrupted. The initial step of autophagy in the pathogenesis of AD is affected, whether it is positive or negative, and it is still controversial. Researchers have shown that some Atg genes are upregulated in AD, but this does not mean induction of autophagy, and this may be due to the lack of other Atg genes leading to insufficient autophagy. Consistent with a decrease in the level of Beclin 1 in the rodent Alzheimer's model, the expression level of Beclin 1 is reduced in AD patients. In the AD mouse model, a decrease in Beclin 1 levels leads to aggregation of Aβ and neurodegeneration.

The pathogenesis of PD is associated with loss of autophagy. The main risk factor for PD may be age, and some genetic defects are also associated with familial PD. The PINK1 and PARKIN can affect selective autophagy to damage mitochondria. PINK1 induces the transport of the E3 ligase into denatured mitochondria, resulting in ubiquitination of voltage-dependent anion channel 1 (VDAC1), which can be recognized by the autophagy regulator p62/SQSTM1 (Geisler et al. 2010). All of these steps are necessary for the removal of aged mitochondria. Mitochondria can cause cell damage and inflammation, which is a major change in neurodegenerative diseases. Another factor that causes PD to occur is due to the α-synuclein degradation disorder. α-synuclein is degraded by CMA and macroautophagy, which explains why elderly people with reduced autophagy pathways are prone to PD (Cuervo et al. 2004; Vogiatzi et al. 2008). Another pathway to degrade α-synuclein is the proteasome pathway. Recent studies have shown that the degradation of α-synuclein appears to be dependent on the state of ubiquitination, suggesting that it is more effective for its clearance by the proteasome pathway. However, in the case of autophagy downregulation, the degradation of ubiquitinated proteins is slowed down due to the aggregation of the autophagy substrate p62/SQSTM1, which inhibits the proteasome pathway and leads to the accumulation of α-synuclein (Geisler et al. 2010).

Neurodegenerative diseases are a multifactorial and complex disease. The effect of monotherapy is not significant, and there is still a need to strengthen the study of anti-neurodegenerative drugs. Natural products should be a good source because natural products have multiple-target effects. Current research shows that phenolic

compounds can exert neuroprotective effects through a variety of important mechanisms, such as regulating the expression of anti-apoptotic factors, inducing different intracellular signaling pathways, anti-oxygen free radicals, inhibiting pro-oxidant enzymes, and regulating mitochondrial function and chelate redox-active metal ions. The molecular pattern of polyphenols for neuroprotection may be mediated, at least in part, by its potent antioxidant and anti-inflammatory effects, or by the regulation of autophagy to clear abnormal protein aggregates (Nabavi et al. 2018).

In addition to polyphenols in natural products, other alkaloids or terpenoid natural products have also been found to have neuroprotective effects, and this protective mechanism includes the regulation of autophagy. Such natural products can, therefore, prevent or treat neurodegenerative diseases.

54.2 Resveratrol

A large number of studies have confirmed that resveratrol has neuroprotective effects, and many studies have reported the anti-oxidation and neuroprotective effects of resveratrol in neurodegenerative diseases. Studies found that resveratrol may have a therapeutic effect on neurodegenerative diseases by promoting the binding of DEPTOR (DEP domain-containing mTOR-interacting protein) to mTOR, thereby inhibiting mTOR and inducing autophagy. Under normal conditions, the activity of mTOR is regulated by DEPTOR. Binding of DEPTOR to mTOR will inhibit the mTOR pathway and help autophagosomes clear toxic Aβ oligomers, thereby protecting nerve cells. In AD patients, the mTOR pathway is overactivated, leading to autophagic dysfunction and thus lack of clearance for Aβ. Excessive Aβ causes a further decrease in the expression of DEPTOR, thereby increasing the expression of mTOR and accelerating the process of neurodegeneration. Resveratrol inhibits the kinase activity of TORC1 and TORC2 complexes by promoting the binding of DEPTOR to mTOR, thereby regulating the activities of two substrates S6K1 and 4EBP1, inhibiting the synthesis of β- or α-secretase, and reducing Aβ.

Huntington's disease can cause damage to neurons in many parts of the brain, including the striatum, mainly caused by the deposition of Huntington's protein (Htt) and the toxicity of dopamine, which interfere with the formation of autophagosomes. In SH-SY5Y cells expressing wild-type and mutant Htt, 100 μM resveratrol treatment restored autophagy, which was damaged by dopamine, increased LC3-II protein expression, increased p62 degradation, and intracellular accumulation of autophagosomes, which is related to its ability to protect Atg4 (Vidoni et al. 2018).

Resveratrol also has protective effects in PD cell models, including SH-SY5Y cells treated with rotenone and PC12 cells expressing synaptophysin in synaptic neurons. Resveratrol can increase the level of LC3-II, which is an autophagy marker, in a dose- and time-dependent way, as well as increased intracellular autophagic vacuoles and activation of AMPK and SIRT1 indicate that resveratrol is mediated by AMPK-SIRT1 and promotes autophagy (Nabavi et al. 2018). Lin et al. conducted a study to confirm that 20 μM resveratrol protected SH-SY5Y cells from rotenone-induced

neurotoxicity and induced autophagy, increased LC3-II, decreased p62 levels, and further study revealed that the ability of resveratrol to induce autophagy in SH-SY5Y cells was HO-1 (heme oxygenase-1) dependent (Lin et al. 2014).

54.3 Curcumin

Curcumin is mainly derived from turmeric and is also abundant in curry. Our laboratory published five articles from 2008 to 2015 to confirm that curcumin has antidepressant and neuroprotective effects. It was confirmed that its antidepressant effect is related to the regulation of 5-HT system and interacts with the 5-HT1A/1B and 5-HT2C receptors. Curcumin protects PC12 cells from corticosterone-induced cytotoxicity and the mechanism involves regulation of the ERK1/2 pathway. The protective mechanism of curcumin on glutamate excitotoxicity in rats is related to the increase of brain-derived neurotrophic factor (BDNF) levels in brain cortex neurons and activation of TrkB and regulation of MAPK and PI3K cascade pathways. Then, we confirmed that curcumin protects neurons from glutamate-induced excitotoxicity by regulating the AKAP79-PKA interaction network. Although we did not pay attention to the regulation of autophagy by curcumin in earlier studies, the mechanism was related to the regulation of autophagy. Subsequently, using vascular endothelial cells, we demonstrated the induction of autophagy by curcumin and its mechanism.

In addition, it has been found that in the rat model of epilepsy, curcumin is used to combat the status of epilepticus induced by lithium-pilocarpine; after 2 weeks, with curcumin 200 and 300 mg/kg/day, LC3-II/LC3-I ratio and expression of Beclin-1 protein were increased (Zhang et al. 2016). Although epilepsy is not considered a neurodegenerative disease, curcumin can be tried for epilepsy and cognitive deficits induced by β-amyloid deposition.

Curcumin has been intensively studied as a therapeutic drug for the treatment of Alzheimer's phenotype after ischemia because of its potent role in promoting hippocampal neurons. Studies have shown that curcumin 5 μM and 10 μM treatment can reduce the level of amyloid precursor protein in SH-SY5Y cells and decrease the LC3-I/II ratio, indicating that curcumin can treat degenerative diseases by regulating autophagy (Jaroonwitchawan et al. 2017).

Because curcumin has low bioavailability and limited use, its lipophilic structural features facilitate the passage of the compound through the blood–brain barrier to brain tissue. We and some laboratory studies have shown low concentrations of curcumin have a good therapeutic effect on neurodegenerative diseases.

Several clinical trials on curcumin and resveratrol have been underway, and these studies have shown their efficacy in protecting or restoring cognitive function. Moreover, curcumin and resveratrol are safe and there are no reports of serious adverse events. However, these findings are currently not a definitive conclusion. In addition, because these compounds have low oral bioavailability, dosage forms and dosages

need to be improved and evaluated. Determining effective doses, improving bioavailability, and better standardizing formulations will ensure that the drug reaches adequate plasma level. In addition, there is a need to conduct a cohort study on different neurological diseases in order to clarify that polyphenols can indeed have beneficial preventive effects in improving the behavior of neurodegenerative diseases (Nabavi et al. 2018).

54.4 Tripterine

Tripterine (Celastrol) is an active natural product extracted from the Chinese medicinal material *Tripterygium wilfordii*. It has been shown to have neuroprotective activity and has a preventive effect on PD. Studies have shown that tripterine can induce autophagy in the neuroblastoma cell line SH-SY5Y by upregulating LC3-II protein expression. At the same time, it was found that the activity of SH-SY5Y cells was significantly increased compared with the rotenone group after treatment with tripterine. It can be assumed that the mechanism by which triptolide can reverse the damage of rotenone-induced SH-SY5Y cells may be related to the induction of autophagy (Deng et al. 2013). In addition, tripterine increased the ratio of LC3-II/LC3 I by 60.92% ($P < 0.001$), suggesting that triptolide activates autophagy. The inhibition of autophagy by 3-methyladenine (3-MA) abolished the cytoprotective effect of tripterine. These results indicate that tripterine can protect SH-SY5Y cell damage induced by rotenone, and the autophagy pathway is involved in the neuroprotective effect of tripterine.

54.5 Paeoniflorin

Paeoniflorin (PF) can regulate acid-sensing ion channels (ASICs), which are ligand-activated ion channels that are sensitive to acidic environments and are highly expressed in mammalian nervous systems. After paeoniflorin was applied to rat chromaffin cell line PC12, LC3-II expression was significantly upregulated, and paeoniflorin also inhibited the aggregation of α-synuclein induced by MPP (+) with acidosis. Paeoniflorin has a significant effect on the regulation of ASICs expression and protein activity. Therefore, it is suggested that the protection of neuropeptides by paeoniflorin may be achieved by regulating the autophagy process and increasing the degradation of α-synuclein (Sun et al. 2011).

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Chapter 55
Regulation of Autophagy in Cardiovascular Diseases by Natural Products

Simeng Gu and Xuejun Li

Abstract Several major cardiovascular diseases, such as heart failure (HF) and atherosclerosis (AS), have been linked to autophagy dysfunction. The influence of autophagy on the occurrence and development of cardiovascular diseases has two sides. Generally, the induction of autophagy at a low level can provide energy and nutrients for cells through degradation of damaged organelles, protect cardiomyocytes and vascular endothelial cells, and stabilize atherosclerotic plaques. However, excessive autophagy may damage cardiomyocytes and vascular endothelial cells and even cause cell death. Therefore, the study on the role and mechanism of autophagy in the pathogenesis of cardiovascular diseases may not only provide new targets for the treatment of cardiac remodeling, myocardial ischemia and reperfusion injury, atherosclerosis and heart failure, but also provide clues for the developing new drugs on prevention and treatment of clinical cardiovascular diseases. In this chapter, we reviewed the research progress on resveratrol, curcumin, epigallocatechin-3-gallate, and cordyceps sinensis on their recent research progress for cardiovascular diseases. Regulating autophagy may be an effective strategy for the treatment of cardiovascular diseases in the future.

Keywords Natural product · Autophagy · Cardiovascular diseases · Multi-target drugs

Autophagy plays an important role in the cardiovascular system, and disorders of it can lead to heart failure, atherosclerosis, etc. Autophagy maintains the homeostasis of cardiovascular-derived cells, like cardiomyocytes, endothelial cells, or arterial smooth muscle cells. Mitochondrial autophagy is an autophagic response specifically to damaged mitochondria. It has been reported that defects of autophagy or mitochondrial autophagy can cause the cardiac degenerative diseases in animals and alter autophagy or mitochondrial autophagy flux which have also been shown to affect outcomes in several cardiovascular diseases, such as myocardial infarction, various types of cardiomyopathy, and atherosclerosis (Bravo-San Pedro et al.

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2017). The effect of autophagy on the development of cardiovascular disease is a two-edged sword. Low-level autophagy can provide energy and nutrients by degrading damaged organelles and protects cardiomyocytes and vascular endothelial cells. However, excessive autophagy can damage or even lead to the death of cardiomyocytes and vascular endothelial cell. Therefore, studying the role and mechanism of autophagy in the pathogenesis of cardiovascular disease can not only provide new targets and mechanisms for the treatment of cardiac remodeling, myocardial ischemia and reperfusion injury, atherosclerosis and heart failure, but also provide clues for the application of drugs, including natural products in prevention and treatment of cardiovascular diseases.

55.1 Resveratrol

Resveratrol has been reported to have several beneficial effects similar to calorie restriction which can provide many benefits on heart. The study has found that 20% caloric restriction or resveratrol alone for 6 weeks failed to induce autophagy, but the combination of 20% caloric restriction and 50 mg/kg/day of resveratrol induced autophagy in the heart of 26-month-old rats and changes the expression of autophagy-related proteins such as Beclin-1, Atg5, P62, and the LC3-II/LC3-I ratio. Although oxidative stress has been thought to induce autophagy, the doxorubicin treatment does not induce autophagy. By observing the level of cardiac apoptosis and the determination of serum creatine kinase and lactate dehydrogenase activity, it was confirmed that the combination of CR and resveratrol induced autophagy while avoiding cardiomyocyte damage caused by doxorubicin (Dutta et al. 2014).

Resveratrol can induce autophagy by activating AMPK, reduce cardiac remodeling after infarction, and maintain cardiac function (Kanamori et al. 2013). Even low concentrations of resveratrol can protect cells, in part by autophagy induction mechanisms. In addition, studies suggest that resveratrol-activated autophagy may be achieved by the expression of Rictor, a component of mTORC2, which is known to activate Akt by phosphorylating Ser 473 of Akt (Gurusamy et al. 2010). There are evidences that atherosclerosis is associated with excessive inflammation of the vessel wall, and that natural compounds with anti-inflammatory properties may be expected to be candidates for treatment. For instance, arglabin is a natural inhibitor of inflammatory bodies that induces autophagy in macrophages, thereby preventing the activation of inflammatory bodies, inhibiting inflammatory responses, significantly reducing inflammation, and improving atherosclerosis (Abderrazak et al. 2015). In another example, in human umbilical vein endothelial cells (HUVECs), resveratrol can reduce vascular endothelial inflammation through autophagy mediated by cAMP-PRKA-AMPK-SIRT1 signal pathway.

However, the oral bioavailability of resveratrol is low because of its metabolism to sulfated and glucose acetaldehyde derivatives, which limits its cardiovascular protective effect (Wenzel et al. 2005).

55.2 Curcumin

Curcumin is a typical multi-target drug which has a multifaceted effect. Our laboratory used human umbilical vein endothelial cells (HUVECs) to observe the effects of curcumin on autophagy and possible mechanisms (Han et al. 2012), and then found that curcumin can induce oxidative stress-injured HUVECs cells autophagy and protected cells. Curcumin can downregulate the PI3K/Akt/mTOR cell signal pathway, promotes the depolymerization of the BECN1/Bcl-2 complex, and induces autophagy. Curcumin inhibits the translocation of FoxO1 into the nucleus during oxidative stress, and knockdown of FoxO1 can inhibit autophagy induced by curcumin. Curcumin can also increase the degree of acetylated FoxO1 in the cytoplasm of HUVECs, enhances the interaction between acetylated FoxO1 and ATG7, and promotes autophagy. In addition, curcumin increases the expression of the autophagosome protein RAB7. Our study suggests that autophagy can protect cells from oxidative stress in vascular endothelial cells. Induction of autophagy may protect cells and reduces apoptosis. This potential mechanism may be the main mechanism of action of curcumin polyphenols on endothelial cell protection and mitigation of oxidative stress damage.

There are also studies that suggest curcumin can alleviate isoproterenol-induced cardiac hypertrophy and fibrosis in rats by inhibiting autophagy and activating mTOR (Liu et al. 2018). Isoproterenol treatment can significantly increase the expression of cardiac hypertrophy and fibrosis marker mRNA in rats. While curcumin (200 mg/kg/d, gavage) can reverse the expression of all these markers, histological analysis showed it can alleviate the interstitial fibrosis caused by isoproterenol. In addition, isoproterenol treatment can significantly decrease mTOR mRNA and p-mTOR protein levels, and significantly increases LC3, Beclin-1 mRNA LC3-II, Beclin-1 protein, and LC3-II/I ratio levels. In addition, curcumin treatment can abolish the effect of isoproterenol on the mTOR/autophagy signaling pathway.

Furthermore, curcumin can also prevent diabetic cardiomyopathy by inducing autophagy and reducing apoptosis. Diabetic model rats were induced by low-dose STZ injection combined with high-fat diet, which showed that the apoptosis of cardiac cells increased, and the autophagy inhibited. And the inhibition of autophagy aggravated the apoptosis death of cardiomyocytes. Curcumin treatment improves cardiac function, which activates AMPK and JNK1, promotes phosphorylation of Bcl-2 and Bim, and thus interferes with their interaction with Beclin-1, thereby promoting autophagy and attenuating apoptosis. In addition, AMPK-mediated inhibition of the mTORC1 pathway may be one of the mechanisms by which curcumin plays a role in the regulation of autophagy in the diabetic state (Yao et al. 2018). Regulating autophagy may be an effective strategy for the future treatment of diabetes-related cardiovascular disease.

55.3 Epigallocatechin Gallate (EGCG)

Epigallocatechin gallate (EGCG) is mainly extracted from green tea. Studies have shown that EGCG treatment can increase the formation of autophagy in LC3-II and primary cultured bovine aortic endothelial cells (BAEC) when the ectopic lipid aggregates. Further studies revealed that EGCG-induced LC3-II formation requires calmodulin-dependent protein kinase β's activation, while its knockdown can significantly inhibit EGCG-induced LC3-II formation, which may associate with intracellular Ca^{2+} overload. In order to determine whether EGCG affects palmitic acid-induced lipid accumulation, the study also observed the autophagic flux of EGCG and the co-localization of lipid droplets and autophagosomes. It was found that EGCG can antagonize the defects of autophagy caused by palmitic acid and significantly reduce the accumulation of lipid droplets caused by palmitic acid. Blocking the degradation of autophagosomes will antagonize the beneficial effects of EGCG on the accumulation of ectopic lipids, indicating that the effect of EGCG is through the inhibition of autophagosome degradation, and the mechanism may be to increase the co-localization of lipid droplets and autophagosomes. When the cells were treated with EGCG and palmitic acid and compared with cells treated with palmitic acid alone, it was found that the co-localization of lipid droplets with LC3 and lysosomes increased dramatically. In summary, these findings suggest that EGCG regulates ectopic lipid accumulation mainly by promoting autophagic flux, suggesting that EGCG may be a potential drug for the prevention of cardiovascular complications (Kim et al. 2013).

In addition, EGCG was found to have protective effects on myocardial ischemia–reperfusion injury in rats, which is related to upregulation of PI3K, promotion of phosphorylation of Akt and endothelial nitric oxide synthase (eNOS), and decreased expression of cleaved caspase-3. Importantly, it also inhibits excessive autophagy induced by ischemia–reperfusion injury, promotes clearance of autophagosomes, reduces LC3-II/LC3-I ratio, downregulates Beclin-1, Atg5, and p62, and restores autophagy flux while upregulating activated cathepsin D and the phosphorylation of mTOR (Xuan and Jian 2016).

55.4 Cordyceps Sobolifera

Cordyceps Sobolifera (CS) is a Chinese medicine with high economic value. It reverses lipopolysaccharide-induced renal dysfunction in rats by inhibiting apoptosis, autophagy, and oxidation. In vitro, CS has protective effects on lipopolysaccharide-induced Madin–Darby canine kidney epithelial cells (MDCK). After the treatment of CS, the expression of autophagy-related proteins such as caspase12, Beclin-1, and GRP78 was increased, and the apoptosis of MDCK cells was found by TUNEL. It indicates that long-term treatment with CS can inhibit LPS-induced stress and tissue damage, and the mechanism may be related to inhibition of LPS-triggered signaling pathway and thereby apoptosis and autophagy (Wu et al. 2011).

55.5 Conclusion

In general, autophagy is the survival response of cells to nutrient or growth factor deficiency, but autophagy can also lead to non-apoptotic programmed cell death. This process is called autophagic cell death or autophagy-associated cell death, and regulation of autophagy is very important. Natural products have multi-target properties, as most of them are extracted from food or herb medicine, their toxicity is relatively lower than that of chemical drugs, so it may bring benefits for the prevention and treatment of complex diseases. Like cancers, they can simultaneously produce the dual effects of promoting both apoptosis and autophagy. The combination with currently available anti-cancer drugs may produce synergistic effects and overcome drug resistance, which is a valuable new attempt in cancer therapy. But there is still a lot of work to do to get natural products into the clinic.

In the future, we need to do extensive works in the following aspects: combined with new biological techniques to conduct in-depth and meticulous research on the mechanisms and targets of natural products, to clarify their signal pathways for autophagy regulation; modification of the chemical structure of existing natural products to improve the pharmacokinetic properties of these compounds; explore effective combination therapy with other drugs; improve drug delivery; and strengthen basic research to the clinical transformation, to promote the application of natural products in prevention and treatment clinical diseases.

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