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I would like to dedicate this book to my dear wife for her patience, intellect and adventurous spirit. Her continued support and ongoing encouragement were great sources of inspiration when I spent time working on this project.

I also dedicate this book to my invaluable teachers and advisors Prof. Majid Sadeghizadeh and Prof. Sirous Zeinali, the pioneers of molecular biology and medicine in Iran.

And finally, I dedicate this book to those who pursue their dreams with passion and determination despite the many daunting hurdles and pressures they face.

Preface

The CSC theory posits tumor development might arise from a rare population of cells that show the stem cell-like properties. This theory was initially formulated approximately 150 years ago. The recent decade studies have tremendously advanced our understanding of the molecular pathogenesis of cancer. CSCs have been demonstrated to underlie resistance to conventional chemotherapeutics resulting in tumor recurrence and poor prognosis. In this context, the need to develop novel approaches for eradicating CSCs in order to inhibit tumor recurrence is considered as a major challenge in cancer treatment. Novel compounds precisely designed to eliminate CSCs or affect their microenvironment, and administered in concert with conventional chemotherapy, can lead to tumor bulk shrinkage and ablate resistance and relapse. Importantly, the number of preclinical investigations and clinical trials examining the potential use of anti-CSC drugs has grown exponentially in recent years. Despite all efforts made to develop CSC-targeted therapy, further investigations to identify the specific biological characteristics of CSCs could help us in better understanding the origin and molecular behavior of cancer. This lays a solid foundation to program and perform a more specific, safe, effective, "personalized," and "targeted" therapeutic plan.

This book *Cancer Stem Cells: Emerging Concepts and Future Perspectives in Translational Oncology* aims to offer a broad framework for obtaining insight into the state-of-the-art knowledge of CSC biology and function and outline novel approaches for targeting CSCs. These revelations highlight the therapeutic implications of these cells in the future of clinical oncology. This book was scrupulously designed and explicitly written being well suited for graduate students, postdoctoral fellows, and all researchers who are studying different aspects of experimental oncology. Ranging from the fundamental concepts to clinical implications, this book is composed of nineteen chapters organized in three parts. The first part is devoted to delving deep into the biology of CSCs. Chapter 1 serves as an emblem of the whole book and presents a quick walk through the concepts. This chapter deals exclusively with CSC hypothesis and tumor heterogeneity models and highlights the importance of targeting CSCs for cancer therapy. Chapter 2 describes the principal mechanisms of tumor progression and metastasis, focusing in particular

on contribution of defined molecular constituents of metastatic niche to CSC physiology. Given that a link has been established between cytokine networks and cancer development, Chap. 3 aims to explore the contribution of key cytokines to the CSC phenotype in terms of survival and maintenance. By delineating the importance of chemokines as major modulators of tumor microenvironment, Chap. 4 details recent findings on the roles of CXCR4/CXCL12 chemokine axis for tumor progression, CSC maintenance, and its potential translation into therapeutic targeting. In Chap. 5, the general features of CSCs and the roles of noncoding RNAs, especially microRNAs and long noncoding RNAs, in the regulation of CSC properties are discussed. In addition, current therapeutic strategies aimed at regulating noncoding RNAs for the purpose of CSC therapy are summarized. As stem cells might be the targets of transformation during carcinogenesis, Chap. 6 provides evidence that stemness pathways are dysregulated due to accumulated mutations and epigenetic alterations. This chapter lends support to the concept that breast carcinogenesis results from dysregulation of self-renewal pathways of normal mammary stem cells. The second part of the book summarizes recent advances in the study of CSCs in different types of solid tumors and hematological malignancies. In Chaps. 7, 8, 9, 10, 11, and 12, authors effectively cover possible mechanisms involved in CSC theory, its markers, and their potential as prognostic or predictive molecules in terms of survival and treatment of selected cancers. Also, Chaps. 13 and 14 explain the importance of leukemic and lymphoid stem cells. Chapter 13 delineates the role played by malignant stem cells in myeloid and B-cell malignancies, and Chap. 14 provides an in-depth analysis to leukemic stem cells in acute lymphoblastic leukemia (ALL). The latter chapter particularizes that identification and characterization of leukemic stem cells and their integration with molecular studies have served as a basis for understanding the early stages of ALL development, identification of putative leukemia-initiating cells, and definition of their heterogeneity and changes during tumor development/progression. The third part of the book aims to offer novel approaches for targeting CSCs. In Chap. 15, authors discuss the signaling paradigm for stemness pathways, identify druggable targets, and present selected preclinical and clinical findings with agents targeting each pathway. Importantly, this chapter considers other disease-specific targeted agents to uncover roadblocks to the success of these anti-stemness agents including financial considerations, development of Multidrug resistance, and on-target adverse effects. Consistent with the notion that targeting the interplay between paracrine signals arising in the tumor stromal and the nearby cancerous cells holds promise for the successful elimination of CSCs, Chap. 16 offers a precise description to the latest findings in the optimization and tailoring of novel strategies designed for eliminating CSCs or affecting their microenvironment and administered in concert with conventional chemotherapy which can lead to tumor bulk shrinkage and ablate resistance and relapse. As chemoresistance is one of the most important hurdles to be overcome for improving long-term cancer patient survival, Chap. 17 discusses multiple mechanisms identified for CSC-associated chemoresistance and introduces epigenetic-modifying drugs and inhibitors designed for resensitizing CSCs to chemotherapeutics. In line with this, Chap. 18 outlines mechanisms that CSCs employ to resist ionizing radia-

tion and therapeutic strategies that are currently being used in the clinic or are in various stages of development for overcoming CSC-associated radioresistance. Finally, Chap. 19 discusses the diagnostic and therapeutic potentials of CSCs and introduces biomarkers in preclinical models and clinical trials to evaluate the therapeutic effectiveness of CSCs.

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Tehran, Iran
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Abbreviations

βAPN	β-Aminopropionitrile
γ-IR	γ-Irradiation
ω-3 PUFA	Omega-3 polyunsaturated

A

ABC	ATP-binding cassette
ACKR3	Atypical chemokine receptor 3
ADAM	A disintegrin and a metalloprotease domain
ADCC	Antibody-dependent cellular cytotoxicity
ADT	Androgen-deprivation therapy
AFP	Alpha-fetoprotein
AhR	Aryl hydrocarbon receptor
AIDS	Acquired immunodeficiency syndrome
AJCC	American Joint Cancer Committee
ALDH	Aldehyde dehydrogenase
ALK	Activin receptor-like kinase
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
AMPK	Adenosine monophosphate-activated protein kinase
ANGPT1	Angiopoietin1
ANKRD28	Ankyrin repeat domain 28
APC	Adenomatous polyposis coli
APH1	Anterior pharynx-defective 1
APL	Acute promyelocytic leukemia
AR	Androgen receptor
Arx	Aristaless-related homeobox
AT1	Alveolar type 1
ATM	Ataxia-telangiectasia mutated
ATO	Arsenic trioxide
ATRA	All-trans retinoic acid
AβPP	Amyloid beta-protein precursor

B

6-BG	O(6)-Benzylguanine
B-ALL	B-cell acute lymphoblastic leukemia
B-CLL	B-cell chronic lymphocytic leukemia
BAA	Bodipy-aminoacetate
BAAA	Bodipy-aminoacetaldehyde
BADJs	Bronchoalveolar duct junctions
BCC	Basal cell carcinoma
BCL-2	B-cell lymphoma protein 2
Bcl-Xl	B-cell lymphoma-extra large
BCNU	Bis-chloroethylnitrosourea
BCRP	Breast cancer resistance protein
BCRP1	Breakpoint cluster region pseudogene 1
BCSC	Breast cancer stem cells
bFGF	Basic fibroblast growth factor
BiCNU	Bis-chloroethylnitrosourea
BiTEs	Bispecific T-cell engagers
BKCa	Calcium-dependent potassium channels
BM	Bone marrow
BMI1	B lymphoma Mo-MLV insertion region 1 homolog
BMDCs	Bone marrow-derived cells
BMP	Bone morphogenetic protein
BPH	Benign prostatic hyperplasia
bsAbs	Bispecific antibodies

C

CACs	Centroacinar cells
CAD	C-terminal activation domain
CAFC	Cobblestone area forming assay
CAFs	Cancer-associated fibroblasts
CAIX	Carbonic anhydrase isoform 9
CALLA	Common acute lymphoblastic leukemia antigen
cAMP	Cyclic adenosine monophosphate
CAN	Copy number alterations
CAPP-Seq	Cancer personalized profiling by deep sequencing
CAR	Chimeric antigen receptor
CARM1	Coactivator-associated arginine methyltransferase 1
CBCs	Crypt base columnar cells
CBP	CREB-binding protein
CCL2	C-C motif chemokine ligand 2
CCR	Chemokine receptor
CCSP	Clara cell secretory protein
CD	Cytosine deaminase
CD34	Cluster of differentiation protein 34
CD133	Cluster of differentiation protein 133

Cdc42	Cell division control protein 42 homolog
CDF	Difluorinated curcumin
CDK2	Cyclin-dependent kinase 2
CEA	Carcinoembryonic antigen
CGRP	Calcitonin gene-related peptide
Chk	Cell cycle checkpoint kinase
CK-20	Cytokeratin-20
CLL	Chronic lymphocytic leukemia
CLMP	Common lymphoid-myeloid progenitor
CLP	Common lymphoid progenitor
CML	Chronic myeloid leukemia
CMP	Common myeloid progenitor
CNAs	Copy number aberrations
CNS	Central nervous system
coA	Coactivator
coR	Corepressor
COX	Cyclooxygenase
CQ	Chloroquine
CRC	Colorectal cancer
CREB	cAMP response element-binding protein
CRLF2	Cytokine receptor-like factor 2
CRPC	Castrate-resistant prostate cancer
CSCs	Cancer stem cells
CSDC2	Cold-shock domain-containing protein C2
CSLCs	Cancer stem-like cells
CTCs	Circulating tumor cells
CTSD	Cathepsin D
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptor
CYP	P450 oxidase

D

DAG	Diacylglycerol
DAPT	N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
DBH	Debromohymenialdisine
DC-TICs	Delayed contributing tumor-initiating cells
DCIS	Ductal carcinoma in situ
DCs	Dendritic cells
DGCR8	DiGeorge critical region 8
Dhh	Desert hedgehog
DLLs	Delta-like ligands
DMAG	Demethoxygeldanamycin hydrochloride
Dn	Dominant-negative
DNER	Delta and notch-like epidermal growth factor-related receptor
DNMT	DNA methyltransferase

DR	Death receptor
DSBs	Double-stranded breaks
Dsh	Dishevelled
dsRNA	Double-stranded RNA
DTCs	Disseminated tumor cells
Dvl	Dishevelled

E

EBV	Epstein-Barr virus
EC	Endothelial cells
ECM	Extracellular matrix
EFNA1	Ephrin A1
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELAM1	Endothelial adhesion molecule 1
EMP	Erythro-myeloid progenitor
EMT	Epithelial to mesenchymal transition
EpCAM	Epithelial cell adhesion molecule
EPCs	Endothelial precursor cells
ER	Endoplasmic reticulum
ER	Estrogen receptor
ERBB2	Erythroblastic leukemia viral oncogene homolog 2
ERG	ETS-related gene
ERK	Extracellular signal-regulated kinases
ESA	Epithelial-specific antigen
ESCs	Embryonic stem cells
ESL-1	E-selectin ligand 1
ETPs	Early thymocyte progenitors

F

FACS	Fluorescence-activated cell sorting
FADD	FAS-associating death domain
FAK	Focal adhesion kinase
FAL1	Focally amplified lncRNA on chromosome 1
FAP	Familial adenomatous polyposis
Fc	Fragment-crystallizable
FGF	Fibroblast growth factor
FIH-1	Factor-inhibiting hypoxia 1
Flt3	FMS-like tyrosine kinase 3
FKHR	Forkhead transcription factor Foxo1
FOX	Forkhead box protein
FOXOA1	Forkhead box O1A
FRb	Folate receptor b
Fu	Fused
FU	Fluorouracil
FUOX	Fluorouracil and oxaliplatin
Fz	Frizzled

G

G proteins	Guanine nucleotide-binding proteins
G-6-PD	Glucose-6-phosphate dehydrogenase
G-CSF	Granulocyte colony-stimulating factor
GAGs	Glycosaminoglycans
GANT	Gli antagonist
GBM	Glioblastoma multiforme
GFAP	Glial fibrillary acidic protein
GM-CSF	Granulocyte macrophage colony-stimulating factor
GNA15	Guanine nucleotide-binding protein, alpha 15
GPCRs	G protein-coupled receptors
GRK	G protein-coupled receptor kinase
GSCs	Glioblastoma stem cells
GSEA	Gene set enrichment analysis
GSH	Glutathione
GSIs	γ-Secretase inhibitors
GSK3β	Glycogen synthase kinase 3β
GTP	Guanosine-5′-triphosphate
GTPase	Guanosine triphosphate hydrolase

H

HA	Hyaluronic acid
HB-EGF	Heparin-binding EGF-like growth factor
HCC	Hepatocellular carcinoma
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
HER2	Human epidermal growth factor receptor 2
Hes1	Hairy enhancer of split 1
HGF	Hepatocyte growth factor
Hh	Hedgehog
HIF	Hypoxia inducible factors
Hip1	Hedgehog-interacting protein 1
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HNRNPK	Heterogeneous nuclear ribonucleoprotein K
HNSCC	Head and neck squamous cell carcinoma
HoxB	Homeobox protein
HOTAIR	HOX antisense intergenic RNA
HPCs	Hematopoietic progenitor cells
HPI	Hedgehog pathway inhibitor
HPV	Human papillomavirus
HR	Homologous recombination
HRE	Hypoxia response element
HSCs	Hematopoietic stem cells
HSP	Heat shock protein

HSPC Hematopoietic stem and progenitor cells
HUVEC Human umbilical vein endothelial cells

I

IAP Inhibitor of apoptosis proteins
IBC Inflammatory breast cancer
IBD Irritable bowel disease
ICAM1 Intracellular adhesion molecule 1
ICD Intracellular domain
ICL Idiopathic $CD4^+$ T-cell lymphocytopenia
IDH1 Isocitrate dehydrogenase 1
IEX-1L Immediate early response gene X-1
Ig Immunoglobulin
IGF Insulin-like growth factor
IGF-IR Insulin-like growth factor-I receptor
Ihh Indian hedgehog
IL Interleukin
IL-1R1 IL-1 receptor 1
IL-1Ra IL-1 receptor antagonist
IL-1Rap IL-1 receptor accessory protein
IM Imatinib mesylate
iNOS Inducible nitric oxide synthase
INXS Intronic BCL-XS-inducing lncRNA
IP3 Inositoltrisphosphat
iPSCs Induced pluripotent stem cells
IR Ionizing radiation
IRAK Interleukin-1 receptor-activated protein kinase
ISCs Intestinal stem cells
ITAC Interferon-inducible T-cell α-chemoattractant
ITD Internal *tandem* duplication
IWPs Inhibitors of Wnt production
IWRs Inhibitors of Wnt response

J

JAGs Jagged ligands
Jak Janus kinase
JNK c-Jun N-terminal kinases

K

kDa Kilodalton
KLF4 Kruppel-like factor 4

L

L1CAM L1 cell adhesion molecule
LDA Limiting dilution analysis
LDH L-Lactate dehydrogenase
LEF Lymphoid enhancer factor

LGR5	Leucine-rich repeat-containing G protein-coupled receptor 5
lincRNAs	Long intergenic ncRNAs
LIP	Liver-enriched inhibitory protein
LMO	LIM-only domain
lncRNAs	Long noncoding RNAs
LOX	Lysyl oxidase
LRCs	Label-retaining intestinal stem cells
LRP	Lipoprotein receptor-related protein
LSCs	Leukemic stem cells
LTC-IC	Long-term culture-initiating cell assay
LTTICs	Long-term tumor-initiating cells

M

M-CSF	Macrophage colony-stimulating factor
mAb	Monoclonal antibody
MALAT1	Metastasis-associated lung adenocarcinoma transcript 1
MAML	Mastermind-like
MAPK	Mitogen-activated protein kinases
MaSCs	Mammary stem cells
MCAo	Middle cerebral artery suture occlusion
MCP2	Monocyte chemoattractant protein 2
MDR	Multidrug resistance
MDR-1	Multidrug resistance protein 1
MDS	Myelodysplastic syndrome
MDSCs	Myeloid-derived suppressor cells
MECP2	Methyl-CpG-binding protein 2
MEK	Mitogen-activated protein *kinase*
MET	Mesenchymal to epithelial transition
MetSCs	Metastatic stem cells
MGMT	Methylguanine-DNA methyltransferase
MGUS	Monoclonal gammopathy of undetermined significance
MHC	Major histocompatibility complex
MI	Myocardial ischemia
MIB	Mindbomb
MIP	Macrophage inflammatory protein
miRNA	MicroRNA
MM	Multiple myeloma
MMP	Matrix metalloproteinase
MnSOD	Manganese superoxide dismutase
MPPs	Multipotent progenitors
MRD	Minimal residual disease
MRP	Multidrug resistance proteins
MSCs	Mesenchymal stem cells
Msi-1	Musashi 1
mTOR	Mammalian target of rapamycin

MTX Methotrexate
MUC1 Mucin-1
Myc v-Myc avian myelocytomatosis viral oncogene homolog

N

N-Cor Nuclear receptor corepressor
NATs Natural antisense transcripts
NBD Nucleotide-binding domain
NCSTN Nicastrin
NEBs Neuroepithelial bodies
NETs Neuroendocrine tumors
NEURL Neuralized
NF-kB Nuclear factor kappa-light-chain-enhancer of activated B-cells
NF1 Neurofibromin 1
Ngn3 Neurogenin 3
NHEJ Nonhomologous end-joining
NHL Non-Hodgkin lymphoma
NICD Notch intracellular domain
NK Natural killer
NMRI Naval Medical Research Institute, Lynch et al. (1937)
NO Nitric oxide
NOD Nonobese diabetic
NOG NOD/Shi-scid/IL-2Rγ^{null}
NRF1 Nuclear respiratory factor 1
NRPs Neuropilins
NSAID Nonsteroidal antiinflammatory drug
NSC Neural stem cells
NSCLC Non-small cell lung cancer
nu/nu Nude *mouse* (Foxn1^{nu}/Foxn1^{nu})

O

OCT Octamer-binding transcription factor
OPN Osteopontin
OS Overall survival
OVV Oncolytic vaccine virus
OX Oxaliplatin

P

P-CYP Copolymer-cyclopamine conjugate
P-DTX Copolymer-docetaxel conjugate
P-gp P-glycoprotein
PACT Protein activator of PKR
PanIN Pancreatic intraepithelial lesion
PARP Poly-ADP-ribose polymerase
PAUF Pancreatic adenocarcinoma upregulated factor
PAX Paired box gene

PBMC	Peripheral blood mononuclear cells
PCa	Prostate cancer
PcG	Polycomb group
PCGF4	Polycomb group RING finger protein 4
PCSC	Prostate cancer stem cells
PDAC	Pancreatic ductal adenocarcinoma
PDGF	Platelet-derived growth factor
PDGFRA	Platelet-derived growth factor receptor A
PDH	Prolyl hydroxylases
PDK1	Phosphoinositide-dependent kinase 1
Pdx1	Pancreatic and duodenal homeobox 1
PEN2	Presenilin enhancer 2
PF	Platelet factor
PG	Prostaglandin
Ph	Philadelphia
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP2	Phosphatidylinositol bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PKR	Protein kinase R
PLC	Phospholipase C
PlGF	Placental growth factor
PMA	Phorbol-12-myristat-13-acetate
PORCN	Porcupine
PPAR	Peroxisome proliferator-activated receptor
PR	Progesterone receptor
Pre-miRNAs	Precursor microRNAs
Pri-miRNAs	Primary microRNAs
PRC2	Polycomb repressive complex 2
PROM1	Prominin 1
PSA	Prostate-specific antigen
PSC	Prostate stem cells
PSEN	Presenilin
Ptch	Patched
PTEN	Phosphatase and tensin homolog
Ptf1	Pancreas-specific transcription factor 1
pTHRp	Parathyroid hormone-related protein
PTP1B	Protein tyrosine phosphatase 1 B
PYK2	Protein tyrosine kinase FAK2

Q

Q-PCR	Quantitative polymerase chain reaction

R

RA	Retinoic acid
RA	Rheumatoid arthritis

Rac	Ras-related C3 botulinum toxin substrate
RANKL	Receptor activator of NF-kB ligand
RAR	Retinoic acid receptor
Ras	Rat sarcoma protein family
RCC	Renal cell carcinoma
RFS	Relapse-free survival
Rho	Ras homolog gene family
RNAi	RNA interference
RNF51	RING finger protein 51
RONS	Reactive oxygen and nitrogen species
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
RTKs	Receptor tyrosine kinases

S

SABR	Stereotactic ablative radiotherapy
SAHA	Suberoylanilide hydroxamic acid
SALL4	Sal-like protein 4
SCC	Squamous cell carcinoma
SCDSF	Stem cells differentiation stage factors
SCID	Severe combined immunodeficiency
SCLC	Small cell lung cancer
SCs	Stem cells
SDF-1	Stromal cell-derived factor 1
SDH	Succinate dehydrogenase
SETDB1	SET domain bifurcated 1
SFN	Sulforaphane
SFRPs	Secreted frizzled-related proteins
SGZ	Subgranular zone
SH	Src homology
Shh	Sonic hedgehog
SHIP	Src homology 2-containing inositol-5′-phosphatase
siRNA	Small interfering RNA
SIRP	Signal regulatory protein
SL-IC	SCID leukemia-initiating cells
SLE	Systemic lupus erythematosus
SLGCs	Stem-like glioma cells
Smo	Smoothened
SMRT	Silencing mediator of retinoic acid and thyroid hormone receptor
SOCE	Store-operated Ca^{2+} entry
SP	Side population
SP-C	Surfactant protein C
SPNs	Solid pseudopapillary neoplasms
SPPs	Signal peptide peptidases
SRS	Stereotactic radiosurgery

SSBs	Single-stranded breaks
SSEA-1	Stage-specific embryonic antigen 1
STAT	Signal transducer and activator of transcription
Stat5-CA	Stat5 constitutively active
SuFu	Suppressor of fused
SVZ	Sub-ventricular zone

T

T-ALLs	T-cell acute lymphoblastic leukemias
T-TACs	Tumor transient amplifying cells
T-UCRs	Transcribed ultraconserved regions
TAAs	Tumor-associated antigens
TACE	Tumor necrosis factor-alpha-converting enzyme
TAMs	Tumor-associated macrophages
TBI	Total body irradiation
TCF	T-cell factor
TCGA	The Cancer Genome Atlas
TDECs	Tumor-derived endothelial cells
TET2	Ten-eleven-translocation gene 2 tumor suppressor
TGF-β	Transforming growth factor-β
TGF-βR	Transforming growth factor-β receptor
Th1	T-cell helper type 1
TIMP-2	Tissue inhibitor of metalloproteinase 2
TK	Thymidine kinase
TKIs	Tyrosine kinase inhibitors
TMD	Transmembrane domain
TMZ	Temozolomide
TNC	Tenascin C
TNF-α	Tumor necrosis factor-α
TR4	Testicular nuclear receptor 4
TRAF	TNF receptor-associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TRBP	Transactivation response RNA-binding protein
TS	Thymidylate synthase
TSGs	Tumor suppressor genes
TSLP	Thymic stromal-derived lymphopoietin
TTL	Time to development of leukemia

U

3′UTRs	3′-Untranslated region
5′UTRs	5′-Untranslated region
UGP2	UDP-glucose pyrophosphorylase 2
uPA	Urokinase-type plasminogen activator
uPAR	Urokinase-type plasminogen activator receptor
UPR	Unfolded protein response
UPRT	Uracil phosphoribosyltransferase

V

VCAM1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VHL	Von Hippel-Lindau
VIP	Vasoactive intestinal peptide
VLA4/5	Very late antigen 4 and 5
vWF	von Willebrand factor

W

WHO	World Health Organization
WIF-1	Wnt inhibitory factor-1
Wnt	Wingless
WS	WHIM syndrome

X

XIAP	X-linked inhibitor of apoptosis protein
Xist	X-inactive-specific transcript

Z

Zeb	Zinc finger E-box binding homeobox

Part I
The Biology of Cancer Stem Cells

Chapter 1
Cancer Stem Cells: A Quick Walk Through the Concepts

Katayoon Pakravan, Mohammad Amin Mahjoub, Babak Jahangiri, and Sadegh Babashah

Abstract Cancer stem cells (CSCs) are a subpopulation of tumor cells hypothesized to be largely responsible for the gene expression heterogeneity that exists within tumors. Since surviving CSCs have the capacity to regenerate tumor deposits, CSC chemoresistance represents an important clinical concern. CSCs have been shown to exploit a number of different mechanisms to exert resistance to chemotherapy. These mechanisms include increased DNA damage response, deregulation of apoptosis pathways, increased efflux transporter expression and increased expression of drug detoxification enzymes. Mounting experimental evidence suggests that successful cancer therapy must be directed against both CSCs and proliferating cells which make up the bulk of the tumor. In this regards, therapeutic approach based on combination of conventional therapies targeting bulk tumor cells and therapeutic strategies that selectively target CSCs would be of value in curing cancer.

Keywords Cancer stem cells • Tumor heterogeneity • Drug resistance • Targeting • Cancer therapy

1 Cancer Stem Cell Hypothesis

The past decade witnessed significant efforts and progresses in the area of cancer stem cell (CSC) research. CSCs, a small subpopulation of cancer cells, are defined by their ability to undergo long-term self-renewal and give rise to differentiated tumor-cell lineages. Dysregulation of stem cell self-renewal has become increasingly accepted as a requisite for the initiation, progression, and therapeutic resistance of cancer. The cancer stem cell hypothesis postulates that cancers are derived from a self-renewing cancer stem cell population that is also capable of initiating/maintaining cancer. According to this hypothesis, cancer stem cells with the unique

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self-renewal ability are tumor-initiating cells that differentiate into non-self-renewing cells that comprise the bulk of the tumor (Lobo et al. 2007; Shipitsin and Polyak 2008; Tan et al. 2006). CSCs have the unique ability to support new growth in xenograft models, whereas other cell populations from the same tumor are unable to repopulate a tumor in the same growth environment. There are mounting evidence supporting this model in hematologic malignancies and solid tumors.

2 Cancer Stem Cells and Tumor Heterogeneity Models

Each tumor is comprised of a heterogeneous collection of cells with different properties and functions. This heterogeneity can be explained by three models. The stochastic model suggests that tumors arise as a homogenous group of cells and that their heterogeneous function occurs as a result of random, stochastic events. The hierarchy model states that a stem-like precursor cell gives rise to a heterogeneous group of cells that differentiate with different biological and phenotypic characteristics. As the cells continue to divide and differentiate a tumor with heterogeneous cell phenotypes emerges with the CSC at the apex of the hierarchy. A more complete model which combines these two theories describes chromosomal instability in the CSC population and extrinsic environmental factors that lead to heterogeneity within the CSC population. This model offers a mechanism for the formation of a primary tumor by CSC growth initiating a new primary tumor. Ongoing mutations within the CSC population drive further mutations and heterogeneity. New random mutation may promote or inhibit rapid cell growth; however, Darwinian selection favors the cells with the highest proliferative capacity and the most oncogenic phenotype (Datta et al. 2013). When a subpopulation of CSC develops metastatic properties, migratory CSCs can then seed distant anatomic locations. Within each site of metastasis, these CSC not only contribute to tumor bulk, but can undergo further mutations creating multiple tumor sites each with its own unique, heterogeneous cell population.

3 Cancer Stem Cell Characteristics

CSCs are thought to have an important role in tumor proliferation, invasion and metastasis. Epithelial to mesenchymal transition (EMT), in which polarized epithelial cells are converted into motile cells, plays an important role in tumor invasion and metastasis (Thiery 2002; Togawa et al. 2011; Yang and Weinberg 2008). There is a growing body of evidence suggesting that the gain of the EMT properties and the appearance of CSCs share biological alterations and cooperate in the development of cancer metastasis, recurrence, and chemoresistance (Mani et al. 2008; Hollier et al. 2009). The EMT process and loss of E-cadherin allows some CSCs to become metastatic and has been associated with tumor metastasis and poor

prognosis (Kim et al. 2009; Mareel et al. 1997). In this regards, CSCs express EMT markers, and induction of EMT in transformed epithelial cells promotes the generation of the cancer stem-like cell population conferring resistance to chemotherapy (Jordan et al. 2011; Krantz et al. 2012; Mani et al. 2008; Wu 2011; Wu and Wu 2009; Yang et al. 2004).

It is likely that a better comprehension of the biological processes altered in cancer could help in reducing morbidity and increasing survival rates of cancer patients, offering new potential therapeutic targets (Bianchini et al. 2008). In particular, understanding the biological mechanisms of cancer development represents a primary aim in order to eradicate the disease (Bianchini et al. 2008; Braakhuis et al. 2005; Forastiere et al. 2001).

Although CSCs have been defined as neoplastic cells, which have features of stemness such as self-renewal, high proliferation abilities, high migration capacity, and drug resistance, there are differences that discriminate cancer stem cells from cancer cells (Bianchini et al. 2008; Braakhuis et al. 2005; Forastiere et al. 2001). Firstly, the self-renewing mechanism in stem cells responds to a feedback system that regulates the number of mature cells and control the cellular division rate, whereas in cancer cells this feedback mechanism is likely to be disrupted. Moreover, CSCs lack the ability to differentiate into mature cells, suggesting anomalous differentiation programs (Bianchini et al. 2008) (Fig. 1.1).

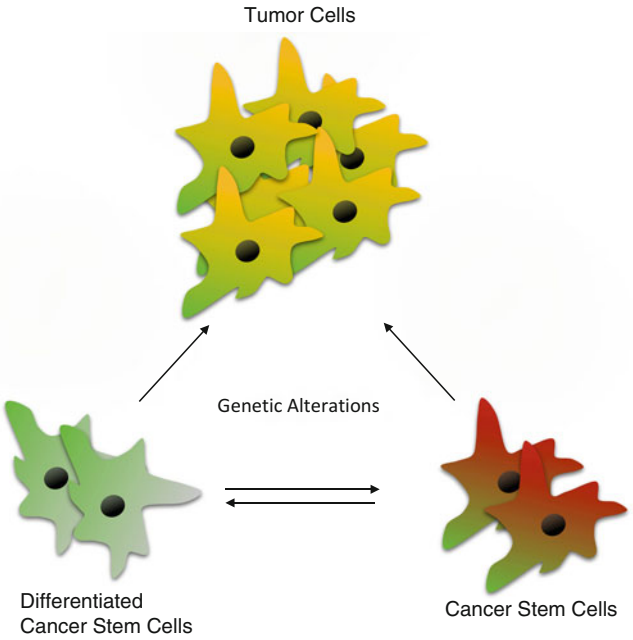

Fig. 1.1 *Cancer stem cells can initiate the development of the tumor bulk*. Genetic alterations could drive the differentiation of cancer stem cells into differentiated cells, still owning stemness characteristics, or into differentiated tumor cellular lines

Moreover, CSCs share several biological properties with their normal counterparts that endow them with a survival advantage upon chemotherapeutic intervention including, dormancy (quiescence), increased DNA repair response, dysregulation of apoptosis pathways, increased efflux transporter expression, and an enhanced reactive oxygen species (ROS) defence capability (Maugeri-Sacca et al. 2011; Zhou et al. 2014).

Several markers for the identification of CSCs have been proposed, including cell surface markers, marker of self-renewal, pluripotency and markers of resistance to therapy. Interestingly, CSCs express related surface markers (such as $CD133^+$, $CD44^+$, $CD166^+$, Aldehyde dehydrogenase, EpCAM/ESA) in various tissue types (Abbott 2006; Marotta and Polyak 2009; Visvader and Lindeman 2008). The main markers and pathways related to CSC characterization are summarized in Table 1.1 and further discussed in next chapters.

4 The Importance of Targeting Cancer Stem Cells

Although the development of cytotoxic chemotherapeutic agents has achieved significant success regarding targeting deregulated pathways and molecular markers in tumor cells, treatment efficacy is markedly reduced due to the emergence of drug-resistance CSC clones. Indeed, patients undergoing conventional chemotherapy, after an apparent remission, often relapse and develop more aggressive diseases. This relies on the fact that CSCs may be responsible for therapy failure due to the specific activated mechanisms which are peculiar to the undifferentiated status of these cells.

As CSCs are dependent on activated stemness pathways such as Notch, Hedgehog and Wnt (Klonisch et al. 2008), targeting key genes that are part of the self-renewal associated signaling pathways could effectively reduce aberrant stem cell renewal in cancer. In this regards, novel approaches focusing on eliminating CSCs or affecting their microenvironment administered in concert with conventional chemotherapy can lead to tumor bulk shrinkage. Such strategies may conduct to the most durable remission and prevent resistance to chemotherapy and radiotherapy (Fig. 1.2). However, targeting only CSCs may not be enough to prevent metastasis or relapse. In this regards, continued development of combination therapies with multiple targets (e.g. targeting CSCs, combination of chemotherapy, differentiation therapy, and targeting microenvironment) would be of value in cancer therapy. The strategies of targeting CSCs are discussed in detail in Part III of this book.

5 Concluding Remarks

Chemotherapy is an important therapeutic strategy for many types of cancer; however, drug resistance remains the main clinical obstacle to cure in cancer, limiting the effectiveness of chemotherapy to eliminate all cancer cells. Currently, resistance

Table 1.1 Cell surface markers and signaling pathways in cancer stem cells

Surface marker/signaling pathway/transcription factor	Characteristics
CD133 (prominin-1, PROM1)	It was first described in human hematopoietic stem cells (Miraglia et al. 1997). The expression of this Cell surface glycoprotein has been described in various types of cancer. $CD133^+$ cells are more resistant to chemotherapy and therefore can evade standard treatments and later repopulate tumor bulk as a mechanism for tumor recurrence (Bertolini et al. 2009)
CD44	It is involved in cellular adhesion, migration, and metastases in certain types of tumors such as breast (Shipitsin et al. 2007), prostate (Collins et al. 2007), pancreatic (Li et al. 2007), and head and neck squamous cell carcinomas (Prince et al. 2007)
CD166 (ALCAM)	Its expression is pathologically correlated with aggressive disease in a variety of cancers and aberrant cell surface CD166 expression is strongly correlated with a shortened survival (Levin et al. 2010; Weichert et al. 2004)
EpCAM/ESA	Epithelial cell adhesion molecule/Epithelial surface antigen that is linked to a more aggressive tumor phenotype (Lugli et al. 2010)
ALDH	A detoxifying enzyme playing a role in the differentiation of stem cells and its activity predicts poorer clinical outcomes (Burger et al. 2009; Ginestier et al. 2007; Huang et al. 2009)
CXCR4	It has been detected in lung, pancreas and prostate CSCs and its overexpression relates to poor prognosis (Bertolini et al. 2009; Hermann et al. 2007; Miki et al. 2007)
CXCL8 and CXCR1	Their expression is associated with pancreatic CSCs and is linked with a lower survival rate due to metastasis of pancreatic cancer cells (Chen et al. 2014)
Notch pathway	It regulates cellular proliferation and differentiation via cell-to-cell communication and has a highly conserved role in determining cell fate during embroygenesis (Insan and Jaitak 2014)
Wnt pathway	It plays a critical role in embryogenesis, development, and stem cell self-renewal. Its overexpression can lead to epithelial and mammary tumors. Deregulated Wnt signaling has been shown in a large variety of cancers including hepatocellular carcinoma, hepatoblastoma, colorectal cancer, acute and chronic myelogenous leukemia, multiple myeloma, gastric cancer, Wilms' tumor, and NSCLC (He et al. 2005)
Hedgehog pathway	Hedgehog pathway is involved in embryogenesis. It controls migration, polarity, differentiation, proliferation, and transformation of progenitor cells (Varjosalo and Taipale 2008)
Transforming growth factor-β (TGF-β)	A family of cytokines inducing EMT, the complex process by which cells down-regulate E-cadherin, lose their adhesive properties and cell polarity, and gain invasive and migratory properties (Massague 2008)
Octamer-binding transcription factor 4 (Oct-4)	It is crucial for embryonic stem cell self-renewal along with Nanog and Sox2 (Chen et al. 2008). Oct-4 is present in high grade tumors and is a poor prognostic marker of lung adenocarcinoma survival (Chiou et al. 2010)

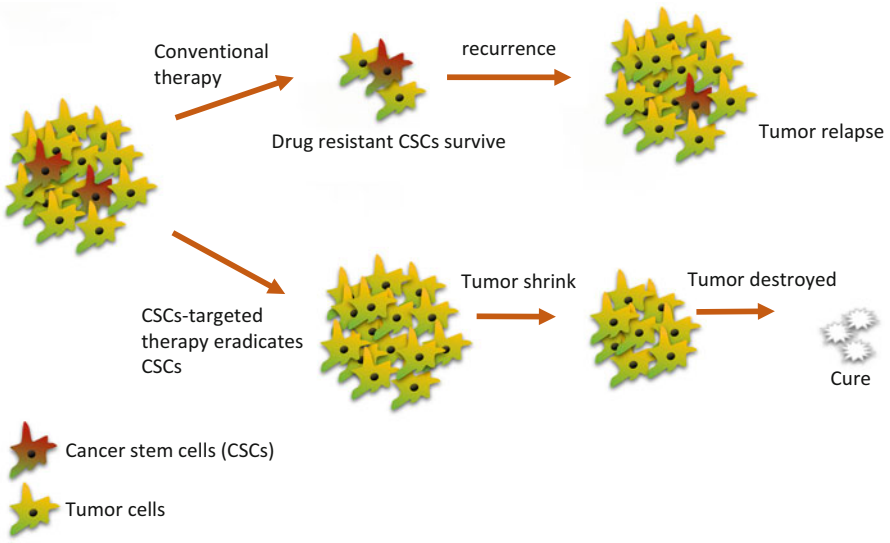

Fig. 1.2 *Cancer stem cells (CSCs) are resistant to conventional therapy*. Conventional therapies such as chemotherapy are directed towards rapidly dividing cells and consequently decreases the number of cancer cells; however, this approach does not target and eliminate CSCs. This leads to relapse of the disease. By targeting CSCs, residual cells are not able to support cancer and undergo apoptosis or differentiation. This strategy may prevent drug resistance and disease recurrence observed in cancer patients

to chemotherapy is believed to cause treatment failure in over 90 % of patients with metastatic cancer and leads to tumor recurrence and poor prognosis of the patients (Longley and Johnston 2005; Abdullah and Chow 2013). When compared to bulk tumor cells, CSCs have a higher intrinsic resistance to chemotherapy, making them the cause of relapse even after achieving a molecular remission.

Although CSCs share a variety of biological properties with normal stem cells such as the capacity for self-renewal, the propagation of differentiated progeny, and the expression of specific cell surface markers and stem cell genes, they differ from their normal counterparts in their chemoresistance and tumorigenic and metastatic activities.

Signaling pathways (such as Wnt, Notch, and Hedgehog) that control self-renewal properties of stem cells are essential for both regulation of EMT/metastasis and self-renewal of CSCs in various cancers (Beachy et al. 2004). Due to the ability to drive tumor initiation and progression, CSCs are considered as potentially useful pharmacologic targets. Recently, there were a surge in the development and clinical evaluation of targeted anti-Notch, anti-Wnt, and anti-Hh agents. However, the convoluted nature and extensive cross-talk between the self-renewal pathways makes identifying appropriate druggable targets difficult. Interestingly, a number of natural compound have shown significant efficacy in inhibiting these stemness pathways. For instance, curcumin, a well-known dietary polyphenol derived from the rhizomes

of turmeric, has the potential to target CSCs through regulation of stemness pathways involved in acquisition of EMT (Bao et al. 2012). In this regards, more clinical trials are required to adequately assess the efficacy and success of these promising agents in cancer chemoprevention and therapy.

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Chapter 2
Cellular Plasticity, Cancer Stem Cells and Metastasis

Paola Ferrari and Andrea Nicolini

Abstract Metastasis is a multistep process that implies genetic modifications and is strongly influenced by the interactions between host and tumor cells, and by tumor microenvironment. Before tumor cells colonize distant organs, they can prepare foreign soil by remotely coordinating a "premetastatic niche" from the primary tumor. The premetastatic niche provides an array of cells, cytokines, growth factors, and adhesion molecules to support metastatic cells on their arrival and to guide metastases to specific organs. Factors secreted by tumor cells, such as VEGF, LOX, IL-6, IL-10, and exosomes, participate in the premetastatic niche formation. Also extracellular matrix (ECM) molecules, namely periostin, tenascin and osteopontin can supply the necessary resources for successful metastatic colonization. One of the key underlying hypotheses of the cancer stem cell (CSC) model proposes that CSCs are the basis of metastases. CSCs in situ may transform to metastatic stem cells (MetSCs) by epithelial-mesenchymal transition (EMT) and subsequently disseminate and form metastatic colonies. Alternatively, MetSCs may derive from disseminated tumor cells that reacquire the competence to initiate tumor growth after a period of indolence. CSCs exhibit properties that are beneficial to metastasize and adapt in the foreign microenvironment, such as mesenchymal characteristics, increased capacity for DNA repair, resistance to apoptosis and to antitumor therapy. Circulating tumor cells (CTCs) are linked to tumor progression in a variety of solid tumors. CTCs are therefore assumed as precursors of distant metastasis. Potentially, a fraction of CTCs have CSC activity; stem-like CTCs may be a critical subset of CTCs with the capacity to form distant metastases. Many therapeutic strategies against CSCs strategies have been investigated. Among them, therapies directed at CSC niche and pre-metastatic niche are of particular interest. These therapies are aimed at targeting vasculature, extrinsic signals and tumor associated macrophages.

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Keywords Metastasis • Epithelial to mesenchymal transition • Niche • Metastatic stem cells • Circulating tumor cells

1 Tumor Progression and Metastasis

Metastasis is a multistep process that allows primary tumor cells to invade the surrounding tissue, intravasate through blood vessels to enter the circulatory or lymphatic system, survive environmental changes, extravasate into new tissue, proliferate at secondary sites and develop a vascular system to support growth (Giaccia and Erler 2008). Different tumor types have the ability to colonize the same or different organ sites (Fidler 2003). Research in this field is identifying genes that support metastasis to particular organs (Yin et al. 1999; Minn et al. 2005a, b; Kang et al. 2003). Another important variable is the temporal course of metastasis. Breast and lung adenocarcinomas typically relapse within a similar range of organs, including bone, lung, liver and brain (Hess et al. 2006). However, breast cancer recurrences are often detected following years or decades of remission (Schmidt-Kittler et al. 2003), whereas lung cancers establish distant macrometastases within months of diagnosis (Hoffman et al. 2000). The temporal gap between organ infiltration and colonization produces a period of metastatic latency (Nguyen et al. 2009).

1.1 Genetic Driven Metastasisation

The genes and activities that underlie the general steps of metastasis can be grouped into several classes, which have been defined as metastasis initiation, metastasis progression and metastasis virulence genes (Chiang and Massagué 2008; Nguyen and Massagué 2007). Metastasis initiation genes allow transformed cells to invade the surrounding tissue, attract a supportive stroma and facilitate the dispersion of cancer cells. These genes could promote cell motility, epithelial to mesenchymal transition (EMT), extracellular matrix degradation, bone marrow progenitor mobilization, angiogenesis or evasion of the immune system (Guo et al. 2008; Tavazoie et al. 2008).

Metastasis progression genes allow cancer cell passage through capillary walls and survival in the newly invaded parenchyma. Metastasis progression genes could have different functions at the primary site and in distant organs. As the structure and composition of capillary walls and the subjacent parenchyma vary in different organs, the functions required for metastatic infiltration, survival and colonization might also differ depending on the target organ. Metastasis virulence genes confer activities that are essential for the metastatic colonization of a certain organ and for which expression becomes detectable only in cancer cells that metastasize to those

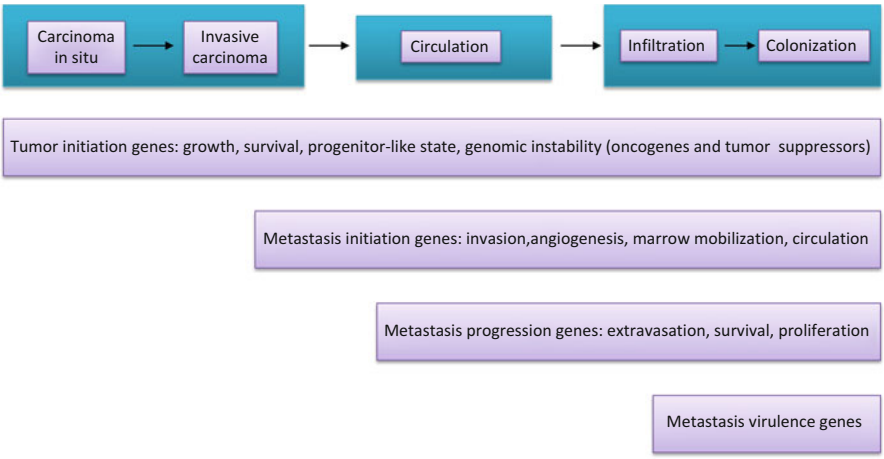

Fig. 2.1 Principal steps of metastasis and hypothetical classes of metastasis genes. Tumor initiation genes include oncogenes as ERBB2, KRAS, PI3K, EGFR, MYC and tumor suppressor genes as APC, TP53, PTEN, BRCA1, BRCA2; metastasis initiation genes include TWIST1, SNAI1, SNAI2, MET, miR-126, miR-335; metastasis progression genes include MMP-1, LOX, ANGPTL4; metastases virulence genes include GM-CSF, IL6, TNF-α

tissues. For example, osteoclast mobilizing factors, such as parathyroid hormone-related protein (pTHRp) and interleukin (IL)-11 do not provide an advantage to breast cancer cells in primary tumors but enable them to establish osteolytic metastases in bone (Yin et al. 1999; Kang et al. 2003; Mundy 2002). The hypothetical classes of metastasis genes are summarized in Fig. 2.1.

1.2 Interactions Between Host and Tumor Cells

Metastasis is strongly influenced by the interactions between host and tumor cells, and by tumor microenvironment. Tumor cells must overcome a different barriers to metastasize, including physical barriers such as extracellular matrix (ECM) and basement membranes, and physiological barriers such as hypoxia and the immune system (Gupta and Massagué 2006). Cells respond to external microenvironmental influences by altering gene expression such as they are able to adapt and survive. The tumor microenvironment thus exerts a selection pressure for cells capable of overcoming these barriers, driving tumor progression and acquisition of metastasis functions.

During preinvasive tumor growth, oxygen and glucose typically can only diffuse 100–150 μm, resulting in portions of the expanding mass becoming hypoxic. Hypoxia selects for cells with low apoptotic potential (Graeber et al. 1996; Erler et al. 2004) and increases genomic instability (Reynolds et al. 1996). Hypoxia also increases the expression of genes involved in glucose transportation, angiogenesis,

anaerobic metabolism, cell survival, invasion and metastasis (Knowles and Harris 2001; Le et al. 2004).

In particular, hypoxia-inducible factors HIF-1α and HIF-2α induce the transcription of over 100 target genes involved in angiogenesis, glycolysis and invasion. Up-regulated angiogenesis genes include vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) that induce blood vessels remodeling. In addition, HIF-α up-regulates matrix metalloproteinase (MMP)-1 and -2, lysyl oxidase (LOX), and the chemokine receptor CXCR4. Degradation of the basement membrane by MMP2 and alteration of the extracellular matrix (ECM) by MMP1 and LOX clears away a barrier to migration. The activation of CXCR4 stimulates cancer cells to migrate to regions of angiogenesis (Bergers and Benjamin 2003; Gatenby and Gillies 2004).

Cancer cells are often surrounded by activated fibroblasts and bone marrow-derived cells (BMDCs). The presence of an inflammatory response in cancer would apply significant selective pressure on the tumor to evade immune-mediated attack. Progressing tumors orchestrate an immunosuppressive environment, a process known as immunoediting (Dunn et al. 2006). Cells involved in chronic inflammation can facilitate tumor formation and progression, mostly mediated by nuclear factor-kB (NF-kB) and cyclooxygenase 2 (COX-2) (Karin 2006; Dannenberg and Subbaramaiah 2003). Tumor-associated macrophages (TAMs) may have tumor-suppressing and tumor-promoting roles. TAMs are stimulated by hypoxia and secrete angiogenesis inducers (including VEGF) and proteases (including MMPs) (Lewis and Pollard 2006; Murdoch and Lewis 2005); TAMs express high levels of HIF-2 transcription factor that is needed for myeloid cell infiltration and activation (Knowles et al. 2004; Cramer et al. 2003). Furthermore, TAMs release growth factors such as PDGF, epidermal growth factor (EGF), hepatocyte growth factor (HGF), which enhance proliferation, survival and invasion (Lewis and Pollard 2006).

TAMs are the main population of inflammatory cells in solid tumors and the cytokines released from them possess diversified significance in tumor development (Lewis and Pollard 2006). TAMs are derived from circulating monocytes and differentiate within the tumor microenvironment (Sica and Bronte 2007; Biswas et al. 2008). The majority of TAMs are M2-like macrophages, with properties that differ from the M1 macrophages, which are usually present in tissue areas with acute inflammation (Lewis and Pollard 2006; Biswas et al. 2008). TAMs generally fail to express pro-inflammatory cytokines for T helper type 1 (Th1) responses but are excellent producers of immunosuppressive cytokines for T helper type 2 responses (Allavena et al. 2008). As TAMs generally exhibit low antigen-presenting and co-stimulating capacity, they ordinarily fail to activate T-cell-mediated adaptive immunity. Therefore the M2- like TAMs are immunosuppressive and facilitate tumor progression (Allavena et al. 2008; Solinas et al. 2009).

Rather than simply suppress the inflammatory response, cancer cells develop mechanisms to both co-opt and perpetuate it. For example, myeloid-derived suppressor cells (MDSCs) are contributing to immunosuppression, but they also facilitate tumor invasion by residing at the invasive front and secreting MMPs. TAMs

are often found at points of basement membrane breakdown and at the invasive front. Growth factors secreted by the TAMs activate fibroblasts; activated fibroblasts become carcinoma-associated fibroblasts (CAFs) and promote primary tumor growth by secreting CXCL12 (chemokine stromal cell-derived growth factor 1, SDF-1), that binds CXCR4 on tumor cells. Angiogenesis is also aided by the action of CAFs through recruitment of endothelial progenitor cells by CXCL12 and by the action of TAMs that are recruited to areas of hypoxia to produce VEGF. In addition to exerting selection for general metastasis-supporting traits, the primary tumor stroma can also select for organ-specific seeding traits. This specificity was recently shown in the case of bone metastatic breast cancer (Zhang et al. 2013). A CAF-rich stroma in breast tumors produces CXCL12/SDF1 and insulin-like growth factor-1 (IGF1), which select for Src hyperactive cancer clones that are superior at responding to these signals with activation of the phosphoinositide-3 kinase (PI3K)/PI3K/protein kinase B (Akt) survival pathway. Src-high clones are thereby primed for seeding the bone marrow where local sources of CXCL12 and IGF1 provide them with a higher chance of survival. As a corollary to these findings, CAF content, CXCL12/IGF1 signaling, and high Src activity in breast tumors all predict an increased likelihood of bone relapse in breast cancer patients (Zhang et al. 2009, 2013).

Cancer cells may leave a primary tumor early and evolve separately from the tumor. It has been proposed that the parallel evolution of early disseminated cancer cells over a period of indolence affords these cells a superior adaptation to their metastatic microenvironment and a leading role in metastatic relapse (Klein 2009). Cancer cell entry into the circulation and lodging in distant organs can certainly occur after minimal genetic changes (Podsypanina et al. 2008; Schardt et al. 2005). However, large-scale genome sequencing studies have shown more similarities than differences between primary tumors and their metastases, suggesting that most of the genetic changes required for metastasis accumulate in primary tumors (Yachida et al. 2010). Actively growing cancer cells in primary tumors may be more likely to undergo variation for the selection of metastatic traits than their precociously dispersed, indolent comrades.

1.3 Epithelial to Mesenchymal Transition and Invasion

Changes in cell-cell and cell-matrix adhesion interactions are necessary to dissociate cancer cells from the tumor (Cavallaro and Christofori 2004). Cell-cell adhesion is mediated primarily by E-cadherin proteins expressed at junctions between cells. Reduced expression of E-cadherin is often observed in aggressive cancers (Friedl and Wolf 2003) and the loss of this protein is highly associated with EMT (Lee et al. 2006). The acquisition of the invasive phenotype has many similarities with EMT, including loss of cell-cell adhesion and increase in cell mobility. During EMT, there is a switch from E-cadherin expression to N-cadherin expression (a mesenchymal cell marker), which promotes cell-matrix adhesion (Lee et al. 2006).

EMT can confer invasive migration capacity to enter circulatory or lymphatic system. Invasive migration involves changes in cell-matrix adhesion and cytoskeleton; cell-matrix adhesion is largely regulated by integrins that bind to specific components of ECM (Guo and Giancotti 2004). Integrins are activated by the contact with specific ECM substrates or through growth factor stimulated signalling (Mitra et al. 2005; Playford and Schaller 2004). Integrin stimulation promotes formation of focal adhesion contacts, focal adhesion kinase (FAK) activation and formation of FAK-Src complexes (Playford and Schaller 2004). Intracellular signaling mediated by FAK leads to actin-myosin contraction and recruitment of MMPs to focal adhesion sites where they degrade ECM (Mitra et al. 2005; Friedl and Wolf 2003).

The basement membrane provides a physical barrier between stroma and epithelial cells. Glycoproteins and proteoglycans provide ligands for integrins, permitting cell orientation and signalling. Tumor cells can overcome the basement membrane by altering their surface receptors such that they can adhere to basement membrane components; for example, tumor cells can increase expression of integrins (that bind laminin and collagen) and CD44 (that permits cell binding to proteoglycans) (Friedl and Wolf 2003; Behrens 1994). In addition, tumor cell can modify the basement membrane composition to facilitate penetration, for example reducing laminin expression. Besides, they can proteolytically disrupt the basement membrane by altering the balance between ECM proteases and their inhibitory proteins; for example, elevated MMP expression is associated with collagen degradation (Morikawa et al. 1988). MMP degradation of ECM generates bioactive peptides, growth factors and cytokines (Egeblad and Werb 2002; Andres et al. 1991; Chakrabarty et al. 1990).

Tumor blood vessels are malformed and irregular and often present breaks that permit the easy access of tumor cells into the circulation; this abnormal vasculature is the result of dysregulated expression of proangiogenic growth factors, inhibition of antiangiogenic pathways, and recruitment of vascular progenitor cells from bone marrow. Tumors do not possess abundant lymphatic vessels. Tumors secrete lymphangiogenic factors such as VEGF-C, but the development of lymphatics is abnormal.

Knowledge of genetic determinants involved in intravasation is limited. Chemoattractant proteins such as chemokines have been proposed to guide cells toward the circulatory system. Tumor cells also move along collagen fibers, a process facilitated by host macrophages (Condeelis and Segall 2003).

EMT genes can be essential for metastasis. In a breast cancer model, inhibition of Twist potently reduced the number of metastatic lesions in the lung. Consistently, inhibiting Twist in either hypoxic cells or in cells overexpressing hypoxia-induced factor (HIF)-1α reversed both EMT and metastasis, and inhibiting Snail decreased metastasis induced by inflammatory signals (Yang et al. 2008). It has been shown a role for Twist in establishing high levels of circulating tumor cells through enhancing intravasation and/or survival in the circulation (Yang et al. 2004). The ability of cells undergoing EMT to intravasate is consistent with observations that EMT occurs at the invasive front of tumors whereby cells lose E-cadherin, detach, invade, and break down the basement membrane. Accordingly, experiments that directly

analyzed EMT and non-EMT cells showed that only the EMT cells were able to penetrate surrounding stroma and intravasate (Tsuji et al. 2009).

A high proportion of distant metastases are differentiated and in some cases metastases can show a greater degree of cellular differentiation than the primary tumors. For example, increased E-cadherin expression in metastases compared to the primary tumors has been reported in human patient specimens (Oka et al. 1993; Kowalski et al. 2003; Chao et al. 2010). Furthermore, the importance of epithelial phenotype in the formation of secondary tumors has been demonstrated in different metastasis models, including bladder cancer (Chaffer et al. 2005, 2006, 2007), prostate cancer (Oltean et al. 2006; Yates et al. 2007), colorectal cancer (Vincan et al. 2007), and breast cancer (Tsuji et al. 2008, 2009; Chao et al. 2010). Hence, both clinical and experimental evidence points to the necessity of disseminated cancer cells undergoing a mesenchymal-to-epithelial reverting transition (MET) in the secondary microenvironment to form macrometastases (Nieto 2013). Consequently, it has been proposed that metastatic cancer cells possess the phenotypic plasticity and acquired EMT-like phenotype for disseminating from the primary tumor, and subsequently a second transition from the EMT-like to MET-like state occurs to facilitate the formation of metastatic tumors at target organs (Brabletz 2012). MET can take part of metastatic formation with tumor cells regaining their epithelial properties at their secondary homing sites (Hugo et al. 2007; Yao et al. 2011). This hypothesis is in accord with the observation that metastatic lesions generally share epithelial features of the primary tumor (e.g., E-cadherin expression) (Chao et al. 2010; Imai et al. 2004).

Tumor cells in the circulatory system are subjected to immune attack, circulatory forces and apoptosis induced by loss of adhesion (anoikis) (Gupta and Massagué 2006). Circulating tumor cells (CTCs) bind platelets that protect them from dangers and increase their chances of survival (Nash et al. 2002; Gasic 1984). Tumor cells also bind thrombin, fibrinogen, tissue factor, fibrin, thus creating emboli (Zhan et al. 2004). These tumor emboli are more resistant to circulatory forces and immune attack (Nash et al. 2002). In the circulation, aggregates of tumor cells associated with platelets are defined as heterotypic clumps. Both CTCs and platelets can express the αvβ3 integrin to promote aggregation of these cells to form tumor emboli (Guo and Giancotti 2004). This aggregation facilitates arrest and can protect against shear forces and natural killer (NK) cell-mediated killing. Activation of αvβ3 integrin can result from CXCL12/CXCR4 signaling and has been shown to be required for formation of tumor emboli and metastasis (Sun et al. 2007; Felding-Habermann et al. 2001).

Platelets have been implicated to actively induce an EMT in circulating tumor cells, either through direct cell–cell contact or secretion of transforming growth factor (TGF)-beta, which is supposed to act in combination with other factors (Labelle et al. 2011). A transient exposure to platelets was shown to be enough for tumor cells to adopt a more mesenchymal state resulting in enhanced invasive and metastatic behavior (Fig. 2.1) (Labelle et al. 2011). One possible implication of platelet-induced EMT in disseminated cancer cells is thus the conversion of these cells to a more stem-like state, which enables them to seed metastasis (see below).

The ability to resist apoptosis is also very important. Loss of cell adhesion can induce anoikis; a variety of receptor tyrosine kinases can confer resistance to anoikis; also tumor emboli formation can promote resistance to anoikis as well (Zhan et al. 2004). Antiapoptosis genes such as BCL2 or BCL-XL, or the loss of proapoptotic genes and genes downstream of the tumor necrosis factor (TNF)-related receptor family, can result in increased metastasis (Martin et al. 2004; Stupack et al. 2006). Part of this may be the result of survival both in the circulation and shortly after extravasation.

Endothelial cells can guard against wandering tumor cells through expression of DARC, a Duffy blood group glycoprotein (Bandyopadhyay et al. 2006). DARC interacts with KAI1 expressed on circulating tumor cells causing them to undergo senescence. KAI1 was originally identified as a metastasis suppressor gene. The immune system can also actively attack circulating tumor cells (Mehlen and Puisieux 2006). For example, NK cells can engage and kill cancer cells via TNF-related molecules such as TNF related apoptosis-inducing ligand (TRAIL) or CD95L. In total, mechanical and cell-mediated stresses can result in a short half-life for CTCs, so their half-life is often as short as a few hours (Meng et al. 2004).

Tumor cell arrest can occur passively through mechanical lodging or can be allowed by cell surface molecules (Arap et al. 1998; Pasqualini et al. 2000). The vasculature of normal tissues where tumor cells extravasate is intact. In normal vessels, endothelial cells are constantly shed from the vessel walls, so creating temporary gaps where tumor cells can attach, as basement membrane components are exposed (el-Sabban ME and Pauli 1994). Vessel wall damage also attracts platelets and tumor cells associated to platelets (Karpatkin and Pearlstein 1981; Karpatkin et al. 1988). Fibrin clots at the site of tumor cell arrest can further attract platelets and circulating tumor cells (Dvorak et al. 1983). Tumor cell arrest can be allowed by P and E-selectin that are expressed by endothelial cells and bind to tumor cells (Kim et al. 1998); tumor glycosylation patterns and cell-cell adhesion molecules such as integrins and CD44 may also have a role (Wang et al. 2004; Ruoslahti 1994; Friedrichs et al. 1995).

VEGF expression by the tumor can also lead to disruptions in endothelial cell junctions and facilitate extravasation of cancer cells through enhanced vascular permeability. This is likely mediated by the activation of SRC family kinases in the endothelial cells (Criscuoli et al. 2005). Expression of hypoxia-induced CXCR4 on CTCs allows for the selective extravasation into certain organs. This selectivity is due to the expression of its ligand CXCL12 by certain organs that include the lung, liver, bone, and lymph nodes (Müller et al. 2001). Also, tumor clump formation facilitates tumor cell arrest by increasing adhesive interactions. ECM components such as fibronectin and laminin enhance tumor cell arrest (Terranova et al. 1984); tumor cells may reside and growth in the intravascular space until they physically break through the vessel. Tumor cells may also extravasate by inducing endothelial cell retraction that permits cell attachment to ECM (Al-Mehdi et al. 2000).

Resumption of cell proliferation at the secondary site needs angiogenesis to supply oxygen and nutrients. The host tissue can influence tumor growth through auto-

crine, paracrine and endocrine signals, and the balance between positive and negative signals determines metastatic proliferation. This can partially explain organ specificity of metastases, as only certain cells can respond to specific proliferation signals (Fidler 2001). For example, IGF-1, hepatocyte growth factor (HGF) and TGF-α are highly expressed in the liver (Zarrilli et al. 1994; Radinsky 1991; Khatib et al. 2005), and cancer cells from colon and breast cancers (that often metastasize to liver) overexpress receptors for these ligands, e.g. epidermal growth factor receptor (EGFR) and c-met receptor (Gross et al. 1991; Radinsky et al. 1995; Bottaro et al. 1991).

The angiogenic "switch" occurs when the ratio of inducers to inhibitors is increased. Inhibitors of angiogenesis include ECM proteins thrombospondin and endostatin (Dameron et al. 1994; O'Reilly et al. 1997); inducers include VEGF, PDGF, basic fibroblast growth factors (bFGF), TGF-β, and ephrin (Steeg 2006). VEGF stimulates endothelial cells, mobilizes endothelial progenitor cells, stimulates outgrowth of pericytes, increases vascular permeability (Senger et al. 1983; Leung et al. 1989); in addition, VEGF is thought to be a key molecule for the homing of VEGFR-positive bone marrow-derived progenitor cells involved in premetastatic niche formation (Kaplan et al. 2005) and for homing of VEGFR-positive tumor cells to metastatic sites (Price et al. 2001).

Tumor cells that have colonized secondary organs are capable of further colonization of other organs. Cells within the metastatic tumor are subjected to similar microenvironmental pressure as the primary tumor, and adapt to overcome the external barriers and seed new terrain. These cells from metastases are able to constantly reseed both primary and secondary tumor (Norton and Massagué 2006). Tumor cells can move multidirectionally, seeding not only distant sites but also their tumors of origin (Comen and Norton 2012). At least in theory, it would seem that compared with uncharted foreign environments or even premetastatic niches, the primary tumor would impose the least resistance to colonization (Karnoub et al. 2007). Support for this concept of tumor self-seeding has recently been provided using mouse model systems and a variety of different cancer types by demonstrating that CTCs can seed the primary tumor and contribute to its mass (Kim et al. 2009). The ability to self-seed is promoted by IL-6 and IL-8, common prometastatic cytokines found in the tumor microenvironment.

2 The Premetastatic Niche: Factors Secreted by Tumor Cells That Affect the Formation of the Premetastatic Niche

Before tumor cells colonize distant organs, they can prepare foreign soil for the subsequent arrival of disseminated tumor cells (DTCs) by remotely coordinating a "premetastatic niche" from the primary tumor (Psaila and Lyden 2009). These niches are often located within distant organs around terminal veins and are characterized by newly recruited hematopoietic progenitor cells of the myeloid lineage and by stromal cells. The premetastatic niche provides an array of cytokines, growth

factors, and adhesion molecules to help support metastatic cells on their arrival, so it is essential for the growth of extravasated tumor cells. An additional function is to guide metastases to specific organs (Kaplan et al. 2005).

Factors secreted by primary tumor cells stimulate mobilization of BMDCs that enter the circulation and reside in sites of future metastases. BMDCs express VEGFR-1 and several other hematopoietic markers including CD34, CD11b, c-kit, and Sca-1, defining them as early hematopoietic progenitors cells engaged with the parenchyma of the distant organ (Kaplan et al. 2005, 2006, 2007; Wels et al. 2008). The key tumor-secreted factors that determine metastatic sites and mediate pre-metastatic niche formation have to be fully identified, although a role of TNF-α, TGF-β and VEGF-A has been demonstrated (Hiratsuka et al. 2006). These factors induce the expression of chemoattractants such as S100A8 and S100A9 by myeloid and endothelial cells, and promote the homing of tumor cells to the premetastatic sites as well as the invasion of circulating tumor cells through a p38-mediated activation of invadopodia (Hiratsuka et al. 2008).

Homing of $VEGFR1^{+}VLA4^{+}$ BMDCs is mediated via the induction of fibronectin, which is a ligand of VLA-4. BMDCs express the VLA-4 (α4β1), thus priming them to sites rich in fibronectin to establish the clusters in preparation for metastasis (Kaplan et al. 2005). It is thought that these cells may become educated within tumors to hunt out and lay the foundations for distant metastasis. Alternatively, the cells may become activated locally or in the circulation due to chemokines secreted by tumor. Secretion of placental growth factor (PlGF), a ligand for VEGFR1 may activate resident organ fibroblasts to synthesize fibronectin, which facilitates the binding of VLA-4 expressing hematopoietic progenitor cells (Peinado et al. 2012).

Tumor cells secrete LOX, an amine oxidase that plays a role in crosslinking collagens and elastins in the ECM, to provoke systemic alterations and induce the formation of the premetastatic niche (Erler et al. 2009). Under hypoxic conditions, breast cancer tumors secrete LOX, which accumulates in premetastatic sites. This favours the recruitment of $CD11b^{+}$ myeloid cells that adhere to cross-linked collagen IV and produce MMP-2, which cleaves collagen and makes it easier for BMDCs and tumor cells to invade the area.

Cancer cells secrete factors such as IL-6 and IL-10 that activate the S1PR1–STAT3 pathway in myeloid cells. This in turn promotes activation of fibroblasts and up-regulation of fibronectin (Deng et al. 2012). Targeting the pro-invasive S1PR1-STAT3 pathway in $Cd11b^{+}$ myeloid cells eliminates de novo formation of premetastatic niches and metastasis, and reduces preformed metastatic niches. In addition, the expression of tissue factor by tumor cells induces the formation in the future metastatic sites of platelet clots, which recruit myeloid cells (Gil-Bernabé et al. 2012.).

Exosomes are another class of tumor derived products which may prime metastases. Exosomes are small secreted vesicles derived from the endocytic pathway. It has been shown that melanoma cells use exosomes to deliver signals that prime the future metastatic sites. These exosomes instruct BMDCs to contribute to tumor growth and metastatic colonization through the transfer of different molecules such as the Met oncoprotein (Hood et al. 2011; Peinado et al. 2012). In renal carcinoma,

microvesicles released from $CD105^+$ CSCs, but not from $CD105^-$ tumor cells, were able to trigger angiogenesis and significantly enhanced the capacity of renal carcinoma cells to metastasize to the lungs (Grange et al. 2011).

MMPs may also play an important role in this process. VEGFR1 signalling is necessary for pre-metastatic induction of MMP-9 expression in endothelial cells and macrophages of the lungs by distant primary tumors (Hiratsuka et al. 2002). Furthermore, stromal derived MMP-2 and MMP-9 have also been shown to contribute to establishment and growth of metastases (Masson et al. 2005). Periostin, tenascin and osteopontin have been previously linked to the induction of angiogenesis in different systems; these ECM proteins are able to regulate VEGF and its receptors and induce angiogenesis. Thus, ECM molecules can supply the necessary resources for successful metastatic colonization and secondary tumor growth (Chakraborty et al. 2008; Tanaka et al. 2004; Shao et al. 2004; Tokes et al. 1999).

3 Characteristics of Cancer Stem Cells That Are Linked to Metastasis

One of the key underlying hypotheses of the CSC model proposes that CSCs are the basis of metastases. To study the role of CSCs in the process of tumor metastasis, Brabletz et al. (2005) suggested the migrating cancer stem (MetCS)-cell concept. They proposed that CSCs in situ can transform to MetCS cells by EMT. Subsequently, the MetCS cells disseminate and form metastatic colonies. MetSC is any DTC that is capable of reinitiating macroscopic tumor growth in a distant tissue. Metastatic stem cells (MetSCs) may already exist in the primary tumor with the necessary traits to overcome the bottlenecks of the metastatic process, or, alternatively, may derive from DTCs that reacquire the competence to initiate tumor growth after a period of indolence (Fig. 2.2).

CSCs exhibit properties that are beneficial to adapt in the foreign microenvironment and eventually form metastasis. Several unique properties necessary for ensuring long life span of normal stem cells may contribute to protection of CSCs in the adverse microenvironment.

3.1 EMT

EMT is characterized by epithelial cells loosening their cell-cell adhesion, losing cell polarity, and gaining the ability to invade and migrate. EMT regulators include Notch and Wnt/β-catenin pathways, TGF-β family members and FGF proteins that serve to set up regulatory networks involving EMT transcription factors such as Snail and Twist. These networks drive morphogenetic movements by repression of the cell-cell adhesion protein E-cadherin, promoting cytoskeletal rearrangement,

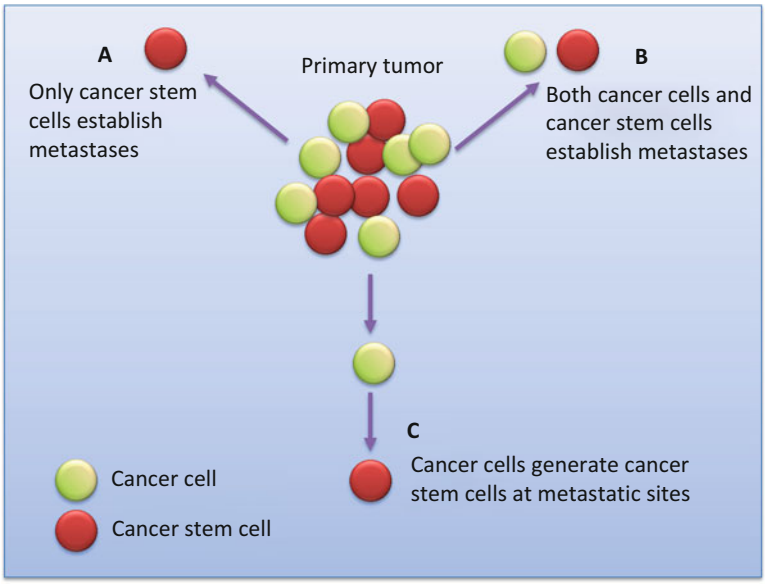

Fig. 2.2 Hypotheses on the origin of cancer stem cells and metastases. *A*. Only CSCs can metastasize to distant tissues; *B*. In animal models of some tumor types, all the cancer cells demonstrate no significant differences in the ability to generate tumors or establish metastases distant sites; *C*. Cancer cells migrate as CTCs from primary tumors to distant tissues, where become CSCs through dedifferentiation

and increasing MMP activity. After cells complete EMT-mediated morphogenetic migration, they can then differentiate into epithelial structures by repressing Snail and undergoing a MET.

CSCs express EMT markers, and induction of EMT in transformed epithelial cells promotes the generation of CSCs (Yang et al. 2004; Mani et al. 2008; Floor et al. 2011; Jordan et al. 2011; Wu 2011; Wu and yang 2011; Krantz et al. 2012). In colon cancer, nuclear accumulation of β-catenin, the feature of Wnt signaling activation and stem cell signaling, is found at the invasive front of the primary tumor (Fodde and Brabletz 2007). Stem-like cells isolated from normal mammary glands and breast tumors also express EMT markers (Damonte et al. 2007; Mani et al. 2008). Overexpression of EMT-inducing transcription factors such as Snail or Twist in transformed mammary epithelial cells increased tumor-initiating frequency in immune-deficient mice (Mani et al. 2008). The mesenchymal phenotype marker Zeb1 may facilitate the acquisition of stem cell-like properties (Peter 2010). Untransformed immortalized human mammary epithelial cells are capable of undergoing an EMT-like state by expressing FoxC2, Zeb factors, and N-cadherin, all of which have been linked to a CSC state (Morel et al. 2008). Likewise, by overexpressing Ras or Her2/neu, a stem-like subpopulation of $CD44^{high}/CD24^{low}$ cells with an enhanced EMT phenotype has been identified (Radisky and LaBarge 2008).

The acquisition of an EMT phenotype may be regulated by signals from the microenvironment or niche. Tumor associated fibroblasts have been shown to enhance the metastatic potential of tumors by promoting migration and extravasation through an EMT process as well as the establishment of a CSC-like state (Aktas et al. 2009; Armstrong et al. 2011; Gregory et al. 2008; Kalikin et al. 2003; Martin et al. 2010).

3.2 Increased Capacity for DNA Repair and Resistance to Apoptosis

Normal stem cells have increased capacity for DNA repair and express higher levels of anti-apoptotic proteins than differentiated cells (Cairns 2002; Potten et al. 2002; Park and Gerson 2005). The enhanced anti-apoptotic and DNA repair capability of CSCs could increase the survival of CSCs for a long period of time under metabolic and/or other environmental stress (e.g., hypoxia) in the target organ and allow them to find adaptive solutions. Autocrine production of cytokines such as IL-4 has been shown to increase anti-apoptotic proteins and induces resistance to therapy-induced cytotoxicity in different cancer types (Conticello et al. 2004).

3.3 Resistance to Anti-tumor Therapy

Many studies have shown that CSCs have increased drug resistance capacity. For example, it has been shown that stem-like subpopulation of cancer cells express high levels of ATP-binding cassette (ABC) transporters that can actively efflux drugs and shield them from the adverse effects of chemotherapeutic insult (Pardal et al. 2003; Lou and Dean 2007; Dean 2009; Donnenberg et al. 2009; Ding et al. 2010; Moitra et al. 2011). There is also growing evidence that CSCs are inherently resistant to radiation (Rich 2007; Debeb et al. 2009; Pajonk et al. 2010; Croker and Allan 2012; D'Andrea 2012). For example, the effectiveness of radiotherapy is mediated by the induction of reactive oxygen species (ROS) in cancer cells. However, it has been found that both human and mouse mammary CSCs contain lower ROS levels than more differentiated tumor cells and accumulate less DNA damage upon radiation (Diehn et al. 2009). Lower ROS levels in CSCs appear to result from increased expression of free radicals scavenging systems (Diehn et al. 2009; Kobayashi and Suda 2012; Shi et al. 2012). The inherent feature of drug resistance in CSCs could activate stress responses to protect them from growth-suppressing conditions in the target organ microenvironment and allow them to persist in foreign tissues for a long period of time.

3.4 Genetic Signatures

Genetic signatures in CSCs are thought to predict tumor recurrence and metastases, providing some support for the concept that CSCs may be metastatic precursors. For example, expression of the CSC marker CD133 in glioblastoma and lung adenocarcinoma is correlated with both the proliferation marker Ki67 and poorer clinical outcomes (Pallini et al. 2008). CD133 antigen expression has also been shown to correlate with patient survival in high-grade oligodendroglial tumors (Beier et al. 2008), rectal cancer (Wang et al. 2009), gastric adenocarcinoma (Zhao et al. 2010), and non-small cell lung cancer (Shien et al. 2012). In patients with colorectal carcinoma, the combination of CD133, CD44, and CD166 can identify patients at low-, intermediate-, and high-risk of recurrence and metastasis (Horst et al. 2009). Methylation of Wnt-target-gene promoters is also a strong predictor for recurrence in colorectal cancer (de Sousa et al. 2011). Finally, the vast majority of disseminated breast cancer cells in the bone marrow displays a CSC phenotype based on CD44 and CD24 expression ($CD44^{+}CD24^{-/low}$) (Balic et al. 2006).

3.5 Experimental Observations on Metastatic Potential of CSCs

The expression of markers such as CD44 or $CD24^{-/low}$ by tumor cells alone does not prove that these cells can generate metastases or that they are necessarily CSCs. Moreover, identification of CSCs within metastatic lesions or in circulating or disseminated tumor cell populations (Balic et al. 2006) does not necessarily mean these cells are capable of establishing disseminated lesions.

Some of the most direct evidence that CSCs establish metastases comes from the demonstration that breast cancer CSCs isolated based upon the putative stem cell markers $CD44^{+}$ and $CD24^{-/low}$ are able to generate primary tumors in an orthotopic site and subsequently produce lung metastases (Liu et al. 2010). In pancreatic cancer models, it has been shown that a distinct subpopulation of $CD133^{+}/CXCR4^{+}$ cells localizes to the invasive edge of tumors and is more migratory than $CD133^{+}/CXCR4^{-}$ cells. Although both populations were equally capable of initiating primary tumor growth, only the $CD133^{+}/CXCR4^{+}$ cells could metastasize to the liver (Hermann et al. 2007). The authors could identify a subpopulation of CSCs that were positive for CD133 and for the stromal-derived factor-1 (SDF1) receptor CXCR4, which showed highly increased migratory abilities. Ablation of these migrating CSCs abolished the capability of pancreatic cancer cells to form metastases (Hermann et al. 2007). In colon cancer different subtypes of CSCs could be identified, one displaying metastases formation abilities (Dieter et al. 2011). Likewise, in inflammatory breast cancer (IBC) a subpopulation of cancer cells displaying stem cell properties was identified as being relevant for metastatic spread (Charafe-Jauffret et al. 2010). Furthermore, the presence of these aldehyde

dehydrogenase-positive cells was suggested to be an independent prognostic factor for early metastasis in patients with IBC (Charafe-Jauffret et al. 2010).

Different populations of CSCs may be responsible for primary vs. secondary tumor sites, implicating CSC heterogeneity as a critical component of this model. Dieter et al. (2011) have demonstrated this heterogeneity within the CSC compartments, reporting at least three phenotypically distinct CSCs in a human colon cancer animal model. To track the contribution of tumor-initiating cell clones, the group generated tumorigenic cells from cancer specimens, marked them with lentiviral vectors, and then sequenced the integration sites in serially transplanted tumors. A population of CSCs was identified as tumor transient amplifying cells (T-TACs) which had limited self-renewal capacity but did form tumors in primary transplants. A second population of CSCs exhibiting extensive self-renewing long-term tumor initiating cells (LTTICs) were able to generate tumors in serial xenotransplants. A third population described as rare delayed contributing TICs (DC-TICs) were exclusively active in secondary or tertiary mice. The marrow could serve as a major source of LT-TICs; however, metastasis formation was predominantly driven by self-renewing LT-TICs (Dieter et al. 2011).

4 The Stem Cell Niche

Similar to normal stem cells, CSCs are thought to reside in a relative stable microenvironment, or niche, in order to retain an undifferentiated state and give rise to more differentiated progenitor cells (Calabrese et al. 2007). The stem cell niche is critical for stem cell self-renewal, survival, function and for maintaining CSC properties (Sneddon 2007).

4.1 The Normal Stem Cell Niche

Normal stem cells in adult tissues reside in specific sites or "niches," the cellular and molecular components of which regulate the self-renewal potential of stem cells and their access to differentiation cues. The location and constitution of stem cell niches have been defined in various tissues, including the intestinal epithelium, hematopoietic bone marrow, epidermis, and brain (Clevers 2013; Hsu and Fuchs 2012; Moore and Lemischka 2006; Morrison and Spradling 2008).

In normal tissues, self-renewal and differentiation of stem cells are tightly regulated, a function fulfilled by the stem cell niche (Morrison and Spradling 2008). For example, the intestinal stem cell resides at the crypt base in close proximity to a secretory non-goblet-like cell type (Sato et al. 2011). These so-called Paneth cells were shown to express components of the various morphogenetic signaling pathways demonstrated to be essential for stem cell maintenance (Sato et al. 2011). Paneth cells support the in vitro outgrowth of $LGR5^{+}$ cells that is one of the cells

thought to be the intestinal stem cell, to organoids. Also the stromal myofibroblasts residing at the crypt bottom provide essential signals for stem cell maintenance (Clevers 2006) and therewith contribute to the stem cell niche. In primary tumors, cancer cells may interact with these native stem cell niches.

4.2 The Cancer Stem Cell Niche

As pathways regulating normal intestinal stem cell biology significantly overlap with those influencing colorectal CSCs, it was hypothesized that the essential stem cell features in tumors are affected by niche cells in equal measure (Medema and Vermeulen 2011), a hypothesis being confirmed by recent studies on colon, pancreatic and brain cancers.

Activin/Nodal signaling molecules, that are essential for sustaining pancreatic CSCs, were shown to not only be provided by the CSCs themselves, but also by stromal pancreatic stellate cells in a paracrine fashion (Lonardo et al. 2011). Similarly, endothelial cells in brain cancers support the CSC state (Borovski et al. 2009). In analogy to normal neural stem cells, $Nestin^{+}/CD133^{+}$ brain CSCs could be located in direct vicinity of endothelial cells (Louissaint et al. 2002). Soluble factors derived from endothelial cells were sufficient to increase the selfrenewing capacity of brain CSCs (Calabrese et al. 2007). In a PDGF-induced glioma mouse model, soluble nitric oxide was identified as the paracrine mediator secreted by endothelial cells and to activate the Notch signaling pathway in a paracrine fashion in glioma CSCs, leading to enhanced tumorigenesis in mice (Charles et al. 2010). Myofibroblasts residing in the tumor microenvironment of colorectal cancer can maintain and even induce a cancer stem-like state through the secretion of HGF, which leads to a boost of the Wnt signaling pathway in adjacent cancer cells (Vermeulen et al. 2010). Interleukin 6 (IL-6), IL-8, and IL-1b directly promote breast CSC self-renewal and survival (Korkaya et al. 2011; Coussens and Werb 2002). The activation of STAT3 by IL-6 through the IL-6 receptor/GP130 complex has been shown to induce breast CSC expansion (Iliopoulos et al. 2009). IL-6 also stimulates the recruitment of mesenchymal stem cells, which produce CXC chemokine ligand (CXCL)-7 to increase the number of breast CSCs in the tumor (Liu et al. 2011b). In addition, breast CSCs express high levels of IL-8 receptor CXCR1, which prevents CSC apoptosis (Ginestier et al. 2010). Also receptor activator of NF-kB ligand (RANKL) has been found to be an important stem cell-stimulating cytokine in the breast (Asselin-Labat et al. 2010; Joshi et al. 2010). The activation of the RANKL-RANK pathway induces EMT and increases the population of CD44high/CD24low CSCs (Palafox et al. 2012). Finally, multiple stromal cell types including carcinoma associated fibroblasts and mesenchymal stem cells can produce prostaglandin (PG)-E2 (Li et al. 2012; Rudnick et al. 2011), that increases the number of aldehyde dehydrogenase (ALDH)-high CSCs through the activation of Wnt/β-catenin signaling (Li et al. 2012). Stem cell niches are sources of developmental and self-renewal signals including Wnt, Notch, the TGF-β family, CXCL12/

SDF1, and hedgehog (Clevers 2013; Hsu and Fuchs 2012; Morrison and Spradling 2008). A source of these signals in the bone marrow are mesenchymal cells that produce CXCL12/SDF1 for hematopoietic stem cell maintenance. The cognate chemokine receptor CXCR4 is frequently overexpressed in bone metastatic cells and provides these cells with chemotaxis and PI3K-mediated survival signals (Müller et al. 2001; Zlotnik et al. 2011).

5 Relationships among Cancer Stem Cells and Pre-metastatic and Metastatic Niches

Establishing CSC niches at distant sites is crucial for the survival of CSCs and is required for the activation of their self-renewal ability for metastatic colonization (Giancotti 2013). There are three distinct sources of metastatic niche functionality: (1) native stem cell niches that metastatic cells may occupy in the host tissues; (2) niche functions provided by stromal cells not belonging to stem cell niches; (3) stem cell stem cell niche components components that the cancer cells themselves may produce (Fig. 2.3).

5.1 Hematopoietic and Perivascular Niche

Cancer cells that infiltrate distant organs may lodge in random locations of the invaded parenchyma. However, recent research provides evidence that cancer cells can occupy native stem cell niches of the host tissue. For example, prostate cancer cells showed affinity for the hematopoietic stem cell niche within the bone marrow, where they may benefit from cues that enhance stem cell properties and deter differentiation (Shiozawa et al. 2011). Using an in vivo micrometasasis model in which DTCs were introduced into immunodeficient mice, it was shown that DTCs target and displace hematopoietic stem cells (HSCs) out of their niche, and establish metastatic foci within the niche space (Shiozawa et al. 2011; Havens et al. 2008).

Another location where cancer cells initiate metastatic outgrowth is around blood capillaries, that is the perivascular niche. This niche has been studied as a preferred residence for glioma stem cells that supplies these cells with hedgehog-, Notch-, and PI3K-activating signals (Charles and Holland 2010; Hambardzumyan et al. 2008). Breast cancer, lung cancer, and melanoma cells that infiltrate the brain conspicuously place themselves around capillaries (Carbonell et al. 2009; Kienast et al. 2010). Perivascular niches may support MetSCs by supplying not only attachment, oxygen, and nutrients but also paracrine factors from the activated endothelium, in what is called “angiocrine” stimulation (Butler et al. 2010). Endothelial cells also express various extracellular matrix (ECM) components that promote metastatic functions in tissue culture (Ghajar et al. 2013). As metastatic lesions grow, the cancer cells recruit TAMs, myeloid precursors, and mesenchymal cells

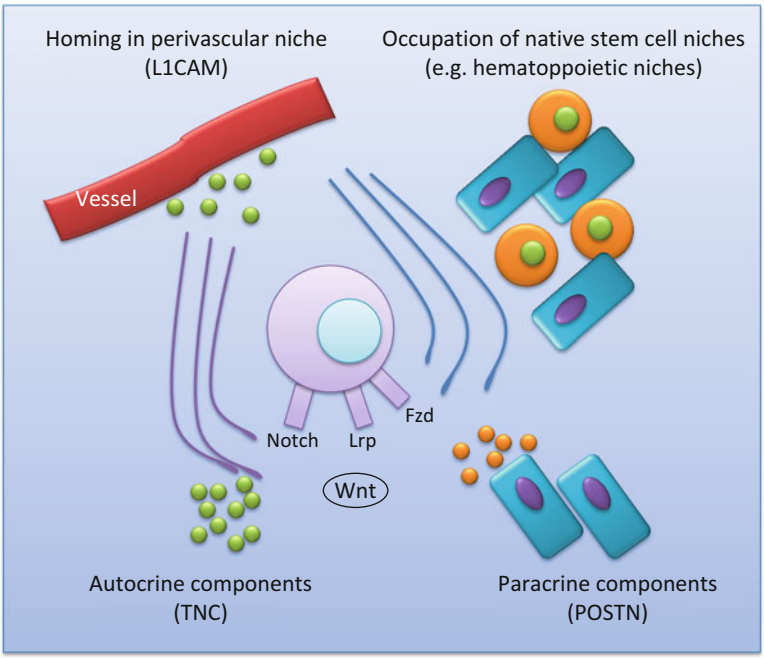

Fig. 2.3 The metastatic niche. Disseminated cancer cells can occupy native stem cell niches (including perivascular and hematopoietic sites) and recruit stromal cells that produce stem cell niche-like components; they also can produce niche components themselves. The supportive niche stimulates Wnt and Notch signalling pathways to increase viability and stem cell expansion. Periostin (POSTN) presents Wnt ligands to Lrp and Frz receptors; tenascin C (TNC) promotes Wnt and Notch signalling. *L1CAM* L1 cell adhesion molecule

that establish paracrine loops feeding back to the cancer cells with various survival and self-renewal factors (Acharyya et al. 2012; Calon et al. 2012; Joyce and Pollard 2009). A recent work showed that brain metastasis-initiating cells express L1 cell adhesion molecule (L1CAM) and use it to stretch over the perivascular basal lamina (Valiente et al. 2014). L1CAM expression in many types of cancer is associated with poor prognosis (Doberstein et al. 2011; Schröder et al. 2009), raising the possibility of a role for L1CAM in metastasis to other organs besides the brain.

5.2 Periostin, Tenascin and VCAM1

Periostin (POSTN) is an extracellular matrix (ECM) molecule highly expressed not only in normal stem cell niches but also in the stroma of the primary tumor and in newly forming metastases. Periostin expression is down-regulated in the adult, except in mesenchymal niches in close contact with tissue-specific stem cells. It was shown that only $CD90^{+}CD24^{+}$ breast CSCs (derived from mouse breast tumors) are

able to seed metastases in the lung after tail-vein injection (Malanchi et al. 2011). CSCs arriving in the lung strongly depended on periostin (POSTN) expression. Infiltrating tumor cells –via secretion of TGFb3– induced POSTN expression in the stromal compartment of the lung. POSTN binds Wnt ligands that signal to the CSCs and maintain their stem-like state. Breast tumors arising in $Postn^{-/-}$ mice led to significantly reduced metastatic burden. Cancer cells stimulate the expression of periostin by stromal fibroblasts; in fact, in the stromal compartment of breast tumors (both human and mouse), POSTN is widely expressed by $aSMA^{+}VIM^{+}$ fibroblasts (Malanchi et al. 2011).

Breast cancer cells also contribute to their own CSC niche by secreting tenascin C (TNC) at metastatic sites; TNC is a hexameric glycoprotein that is found in stem cell niches and supports stem cell functions (von Holst 2008). TNC expression in breast tumors is associated with increased risk of lung metastasis (Minn et al. 2005b). In xenotransplantation models, breast cancer cells that express high levels of TNC have a distinct advantage at initiating metastases after extravasating in the lungs (Oskarsson et al. 2011). TNC enhances Notch and Wnt signaling in the cancer cells. Each of these pathways have been shown to be critical for metastasis and in stem cell biology (de Sousa et al. 2011; Duncan et al. 2005; Malanchi et al. 2011; Reya and Clevers 2005). By expressing their own TNC, breast cancer cells have a higher probability of surviving during micrometastatic outgrowth. Myofibroblasts and $S100A4^{+}$ fibroblasts eventually migrate into the growing lesion to provide additional sources of TNC (O'Connell et al. 2011; Oskarsson et al. 2011).

TNC and periostin thus enhance Wnt and Notch signaling to promote the fitness of MetSCs during the initiation of metastatic colonization. TNC and periostin bind to cell surface integrins and bind tightly to each other (Kii et al. 2010). The physical interaction of TNC and periostin in the ECM may underlie a functional cooperation of these two proteins in stem cell niches (Oskarsson and Massagué 2012). Periostin and TNC in the case of Wnt and Notch signaling, like Src and vascular cell adhesion molecule-1 (VCAM1) in the case of PI3K-AKT signaling, act as amplifiers of the ability of MetSCs to respond to limiting levels of stromal Wnt and Notch ligands for activation of vital self-renewal pathways. The expression of VCAM-1 on cancer cells allows them to interact with macrophages and monocytic osteoclast progenitors via integrin a4b1. This interaction activates PI3K/Akt-mediated survival signals in cancer cells and promotes their osteolytic expansion (Lu et al. 2011; Chen et al. 2011).

5.3 Tumor Dormancy and CSCs

A major limiting step in metastasis is acquiring the ability to sustain growth within a distant site after extravasation. Many cancers such as breast and prostate will not give rise to metastasis until years or even decades after eradication of the primary tumor. Experimentally, it has been shown that the vast majority of extravasated cancer cells do not form macrometastasis (Chambers et al. 2002). These observations of latency are referred to as metastatic dormancy.

Dormant cells are frequently observed in prostate, melanoma and breast cancer (Crowley and Seigler 1992; Demicheli et al. 1996; Van Moorselaar and Voest 2002) and often reside in the lungs, liver and bone marrow. These micrometastases represent a minimal residual disease that results from the inefficiency of metastasizing tumor cells to colonize organs properly following extravasation (Luzzi et al. 1998). Incompatibilities between tumor cells and their tissue soil as well as inability of tumor cells to generate sufficient angiogenesis may result in cell cycle arrest and dormancy (Townson and Chambers 2006). Genes and pathways controlling metastatic dormancy are largely unknown and are important to identify, as they represent a metastatic tumor suppressor mechanism.

Most DTCs detected in bone marrow are proliferatively quiescent, or "dormant" (Müller et al. 2005). Although entry into G0 has been regarded as a failure of cancer cells to proceed with their tumor-propagating potential, it may represent a defense under adverse conditions (Barkan et al. 2010; Klein 2011). Isolation and reimplantation of dormant cells can generate primary tumors, demonstrating that these cells are viable (Luzzi et al. 1998; Naumov et al. 2002; Goodison et al. 2003). Growth of these cells can be activated by angiogenesis or removal of primary tumor, suggesting that limited levels of growth factors or cytokines may induce this dormant state (Holmgren et al. 1995). Unlike the active stroma in primary tumors, the distant tissue where disseminated tumor cells arrive tends to have a more quiescent microenvironment and these quiescent signals may force DTCs into dormancy. For example, abundant bone morphogenetic protein (BMP) ligands in the lung parenchyma inhibit CSC self-renewal, thereby causing metastatic dormancy. Expression of a BMP antagonist, Coco, promotes tumor-initiation ability and allows DTCs to reactivate and colonize (Gao et al. 2012).

A single dormant cancer cell or a dormant micrometastasis can turn into clinically detectable metastasis through an increased secretion of angiogenic factors in the metastatic niche to promote the recruitment and formation of new blood vessels (Takahashi and Mai 2005; Gao et al. 2008; Garcia and Kandel 2012). It has been reported that CSCs promote tumor angiogenesis by actively secreting angiogenic factors such as vascular endothelial growth factor (VEGF) (Bao et al. 2006b; Seton-Rogers 2011). Dormant tumor cells were found to reside in microvasculatures, where quiescent endothelial cell-derived thrombospondin-1 induces tumor dormancy. Upon the induction of neoangiogenesis, the sprouting vasculatures produce active TGF-b1 and POSTN, two important CSC niche signals, to promote metastasis outgrowth (Ghajar et al. 2013).

Mechanisms that contribute to cellular dormancy may relate to the balance between the RAF/MAP kinase kinase (MEK)/mitogen-activated protein kinase (ERK) pathway and the p38-mitogen-activated protein kinase (MAPK) pathway. Inhibition of the former and activation of the latter is associated with cellular quiescence in a G0-G1 state, and the exact balance between the two may depend on cross-talk between the tumor and the microenvironment. Genes that may be important in blocking productive cross-talk between dormant metastasis and its microenvironment include metastasis suppressor genes such as NME23, MKK4, and RKIP (Aguirre-Ghiso 2007; Dangi-Garimella et al. 2009).

6 Relationships between Circulating Tumor Cells and Cancer Stem Cells

A number of studies have linked circulating tumor cells (CTCs) to tumor progression in a variety of solid tumors, and CTC enumeration has begun to be utilized as a prognostic tool in patients with metastatic breast (Cristofanilli et al. 2004), colon (Cohen et al. 2008) and prostate cancer (Danila et al. 2007). These cells are therefore assumed to be a surrogate marker of minimal residual disease and precursors of distant metastasis.

Despite the prognostic relevance of tumor cell dissemination, detection of tumor cells in blood is not necessarily followed by relapse of disease. While most of these cells are already apoptotic or dead and others will successfully be eliminated by shear forces of the bloodstream, only a small group of CTCs possesses the ability to extravasate and migrate through the endothelial cell layer (Frisch and Screaton 2001; Cameron et al. 2000; Sleeman et al. 2011). Merely a fraction of those is able to survive at secondary sites and cause tumor growth "metastatic inefficiency" (Méhes et al. 2001; Larson et al. 2004).

6.1 CTCs with CSC/EMT Phenotype

Whether CTCs are simply associated with disease worsening or whether they directly contribute to metastatic progression remains to be determined. Potentially, a fraction of CTCs have CSC activity, and it is hypothesized that CSCs in a primary tumor which enter the circulation become circulating CSCs and remain so until they lodge or home to a target organ. If true, then stem-like CTCs may be a critical subset of CTCs with the capacity to form distant metastases. If the spread of CSCs leads to metastasis, then it would be expected that some CTCs would express stem cell markers (Aktas et al. 2009; Kasimir-Bauer et al. 2012). Markers useful in the isolation and characterization of CTCs are shown in Table 2.1.

A study identified CSCs in a CTC population among breast cancer patient peripheral blood samples. This study showed that among a total of 1439 CTCs, 66.7 % of patients showed a putative stem cell/progenitor phenotype (35.2 % $CD44^{+}/CD24^{-/low}$ or 17.7 % $ALDH1^{high}/CD24^{-/low}$) in CTCs; 35 % of the CTCs in 20

Table 2.1 Markers used in the isolation and characterization of CTCs

Detection and enrichment markers	Epithelial markers	Mesenchymal markers	Stem cell markers
Cytokeratins 8, 18, 19, EpCAM, EGFR, HER2, MUC-1	Cytokeratins 8, 18, 19, E-cadherin, EGFR, EpCAM, HER2, MUC-1, pan-Cytokeratin	Akt2, N-cadherin, Fibronectin-1, FoxC2, Serpine-1, Slug, Snail-1, Twist-1, Vimentin, ZEB-1, ZEB-2	ALDH1, CD133, CD24, CD44, Bmil

out of 30 patients exhibited the BCSC $CD44^{+}/CD24^{-/low}$ phenotype, whereas 17.7 % of the CTCs identified in seven patients were $ADLH1^{high}/CD24^{-/low}$ (Theodoropoulos et al. 2010).

Metastasis initiating cells containing CTC populations originating from primary human luminal breast cancer expressing epithelial cell adhesion molecule (EpCAM), CD44, CD47, and MET caused lung, liver, and bone metastasis in mice. In a small patient cohort exhibiting tumor metastasis, the population of EpCAM+CD44+CD47+MET+ correlated with increased metastasis and low overall survival (Baccelli et al. 2013). CTCs obtained from patients with Dukes' B and C colon cancers were shown to express CD133, carcinoembryonic antigen (CEA) and cytokeratin. Prognosis among these patients is significantly poorer due to metastasis than those individuals who were found not to express these markers in their CTCs (Pantel and Alix-Panabières 2007). Circulating CSCs were detected in the blood of patients positive for colonic adenocarcinomas. Authors isolated a relatively pure population of CSCs ($CD45^{-}/CK19^{+}$), free of red blood cells and largely free of contaminating $CD45^{+}$ white blood cells. Enriched circulating CSCs from patients with colon adenocarcinomas had a malignant phenotype and co-expressed CSC markers (DCLK1/LGR5) with CD44/Annexin A2. CSCs were not found in the blood of non-cancer patients, free of colonic growths. Enriched circulating CSCs from colon cancer patients grew primary spheroids, suggesting the presence of tumor-initiating cells in the blood of these patients (Kantara et al. 2015).

In a human-to-mouse xenotransplantation experimental model, viable tumorigenic melanoma CTCs were isolated and it was demonstrated that they were capable of metastasis formation. The detection of melanoma CTC in human-to-mouse s.c. tumor xenotransplantation models correlated significantly with pulmonary metastasis formation. Moreover CTCs isolated from murine recipients of s.c. melanoma xenografts were capable of primary tumor initiation and caused metastasis formation upon xenotransplantation to secondary murine NOD-scid IL2Rγ (null) recipients. These results provide initial evidence that melanoma CTC are tumorigenic and demonstrate that CTC are capable of causing metastatic tumor progression (Ma et al. 2010).

It has been recently postulated that EMT plays a key role in the process of tumor cell dissemination (Kasimir-Bauer et al. 2012; Giordano et al. 2012; Barrière et al. 2012; Aktas et al. 2009). Tumor cells undergoing EMT may migrate into peripheral blood as CTCs; due to their mesenchymal stemness features, these cells might be able to reach distant sites of the body and initiate metastases. Loss of E-cadherin, overexpression of N-cadherin, and cytoskeletal alterations (e.g., expression of vimentin) are the hallmarks of EMT. So far, defining the CSCs in a population of CTCs has proven extremely challenging given current limitations in the capture of CTCs (Monteiro and Fodde 2010). CTCs seem to represent a highly heterogeneous cell population with regard to their morphology, molecular characteristics, implantation efficiency after dissemination and their metastatic potential (Lianidou et al. 2013; Fehm et al. 2010).

6.2 Stemness and EMT Identification in CTCs

The challenge in identifying and detecting CTCs is based on their rare number as well as the lack of a universal marker. The majority of methods are based on the detection of epithelial markers, and cells undergoing EMT or with a mesenchymal phenotype might thus be missed. Only a few markers useful in the isolation of CTCs with a mesenchymal phenotype have been evaluated.

In the past 10 years, the number of assays to detect and characterize has increased. Due to the low frequency of the isolated tumor cells, all techniques have to be extremely sensitive. In several cases the first step is the enrichment of tumor cells (Ross et al. 1993). The choice of enrichment and characterization steps depending on the markers analyzed (especially EpCAM) is crucial to allow as well as to limit the detection of cells undergoing EMT or not. One way to enrich disseminated tumor cells is density gradient centrifugation. Due to the lack of a general marker, tumor cells are characterized as epithelial cells which are positive for EpCAM or cytokeratins (Fehm et al. 2002). Another way to enrich CTCs is to label the cells with specific antibodies which are conjugated with magnetic particles. Several tests are based on the immunomagnetic enrichment of epithelial markers, especially EpCAM (Cristofanilli et al. 2004; Fehm et al. 2009), therefore limiting the possibilities to detect mesenchymal tumor cells which have undergone EMT. Tests differ in the subsequent characterization of the CTCs.

The semiautomatic CellSearch system (Janssen Diagnostics, Raritan, NJ, USA) which has been approved by the FDA is based on an immunomagnetic enrichment of epithelial cells using EpCAM-specific antibodies coated with magnetic beads. CTCs are quantified and further characterized by immunofluorescence detecting cytokeratins (CKs) 8, 18, and 19 and CD45 to exclude leucocytes as well as staining of the nuclei (DAPI) (Cristofanilli et al. 2004, 2005). Additional staining of the CSC marker CD44 can be made (Lowes et al. 2012).

In the AdnaTest Breast Cancer (AdnaGen GmbH, Langenhagen, Germany) this enrichment step is performed using magnetic beads which are coated with EpCAM- and mucin-1 (MUC1) specific antibodies. The additional characterization of the CTCs is made by detection of the EMT and stem cell markers TWIST, Akt2, PI3K, and ALDH1, respectively (Kasimir-Bauer et al. 2012; Aktas et al. 2009).

Several approaches to enrich CTCs use special chips combining microfluidics and immobilization of CTCs by binding of specific antibodies (e.g., CTC-chip, Herringbone Chip) (Nagrath et al. 2007; Stott et al. 2010). The latter chip was used by Yu et al. (2013) to establish an RNA in situ hybridization assay to detect and quantify CTCs with either an epithelial or mesenchymal phenotype or with a phenotype in between (partial EMT). The expression levels of seven epithelial transcripts (EpCAM; CK 5, 7, 8, 18, and 19 and cadherin 1) and three mesenchymal transcripts (SERPINE1/PAI1, cadherin 2, and fibronectin 1) were analyzed to characterize CTCs which were detected by binding at least one of the following antibodies: EpCAM, HER2 or epidermal growth factor receptor 2 (EGFR). Flow cytometry is another technique which allows an individual characterization of rare cells like

CTCs. Using flow cytometry, Giordano et al. (2012) could detect a subpopulation of cancer stem cells expressing either ALDH1, CD44, or low amounts of CD24 or ALDH1 and CD133. Although the majority of assays use EpCAM as detection marker, different markers are currently used to detect and enrich CTC. As CTCs change their phenotype during EMT and MET, false negative results can be obtained depending on which detection marker was used. EpCAM-based assays involve the risk that CTC showing a mesenchymal phenotype might be missed.

The hypothesis that EMT markers can be detected among the CTCs of breast cancer patients has been confirmed by various studies in both metastatic and early breast cancer (Giordano et al. 2012; Barrière et al. 2012; Aktas et al. 2009; Mego et al. 2012a, b; Armstrong et al. 2011; Kallergi et al. 2011; Raimondi et al. 2011). EMT markers positive CTCs can be detected in up to 26 % of metastatic breast cancer patients. Moreover, a high expression of EMT markers predicted shorter progression free survival in these patients (Mego et al. 2012b). Aktas et al. (2009) showed in 39 metastatic breast cancer patients that EMT markers, such as Twist1, Akt2, and PI3Kα, can be co-detected in up to 62 % of CTC positive blood samples; EMT markers were more likely to be found in patients resistant to therapy, suggesting increased invasiveness of tumor cells undergoing this process. In primary breast cancer EMT markers could be detected in 72 % of CTC positive and 18 % of CTC negative patients, respectively (Kasimir-Bauer et al. 2012). Expression of EMT markers (e.g., vimentin, fibronectin) was found in up to 38 % of all stage breast cancer patients tested by the standard definition as CTC negative (Raimondi et al. 2011).

These findings suggest that, in addition to CTCs expressing epithelial antigens, a fraction of CTCs with exclusively mesenchymal phenotype could exist and thus remain undetectable for assays based on epithelial character of these cells. However, due to the methodology, morphological features of the cells were not evaluated in these trials (Aktas et al. 2009; Kasimir-Bauer et al. 2012). In this regard, CTCs coexpressing mesenchymal and epithelial markers have been visualized in three other studies in breast cancer patients confirming that both kinds of markers can be expressed in the same cell (Yu et al. 2013; Armstrong et al. 2011; Kallergi et al. 2011). Additionally, Vimentin positive CTCs were detected in peripheral blood of metastatic breast cancer patients while paired metastases from the same patients were shown to be negative for this marker (Armstrong et al. 2011). This suggests a reversibility of the EMT process once tumor cells reach their destination resembling the phenomenon of epithelial plasticity known from embryonic development (Nieto 2013).

In a recent study by Kasimir-Bauer et al. (2012) on 502 primary breast cancer patients 46 % of CTC positive and 5 % of CTC negative blood samples were positive for ALDH1, a common stem cell marker. Similar findings have been shown by Aktas et al. (2009) in the metastatic situation. Moreover, a presence of stem cell-like CTCs in peripheral blood of breast cancer patients was shown to be associated with therapy resistance; stem cell markers or EMT factors or both were detected in 74 % (25/34) of nonresponders and in 10 % (2/21) of patients who responded to systemic

treatment. In the trial by Raimondi et al. (2011), an overexpression of stem markers in CTCs was correlated with advanced stage of disease.

7 Principal Therapeutic Strategies Against Cancer Stem Cells: Focusing in Particular on Metastatic Niche and Microenvironment as Potential Therapeutic Targets

To prevent disease relapse and achieve permanent cure, the CSCs that sustain tumor growth must be eradicated in addition to killing the bulk cells of the tumor. However, properties of CSCs, such as quiescence or expression of drug-resistance transporters, may make them difficult to eliminate using conventional cytotoxic drugs that kill the bulk tumor cells. It will be crucial to understand the unique biology of CSCs in order to develop novel treatments that effectively target these cells.

There are several obstacles to be overcome in the development of effective CSC-targeted therapies. Such treatments must be selective for CSCs and spare normal stem cells. There is recent evidence in acute myeloid leukemia that the pathways that regulate self-renewal in normal stem cells are not completely abolished in leukemia stem cells (Hope et al. 2004). Thus, drugs that target critical processes in CSCs, such as survival or self-renewal, may prove intolerably harmful to their normal counterparts. Furthermore, CSCs will likely have acquired genetic or epigenetic changes that allow them to bypass normal tumor-suppressing processes such as senescence or apoptosis in response to DNA damage, and CSCs are believed to be more resistant to chemotherapy than more differentiated cancer cells. Treatment with agents that normally induce senescence or apoptosis may actually provide a growth advantage to CSCs (Bao et al. 2006a). Ideally, effective therapies will target pathways that are necessary for CSC survival but not for the survival of normal stem cells. Here the principal therapeutic strategies against CSCs are summarized (Fig. 2.4).

7.1 Directly Targeting CSCs via Surface Markers

CD133 is a well characterized marker for putative cancer stem cells (Wu and Wu 2009). Blockage of CD133 reduced the capacity of the melanoma to metastasize (Rappa et al. 2008), suggesting that CD133 might be a potential therapeutic target for CSCs in melanoma and other cancer types (Wu and Wu 2009).

CD44 is a marker of CSCs and also an adhesion receptor involved in metastasis and drug-resistance. Inhibition of CD44 using a siRNA decreases cancer cell adhesion to bone marrow endothelial cells in prostate and breast cancer cell lines (Draffin et al. 2004). A CD44v6-targeting immuno-conjugate, bivatuzumab mertansine, has been evaluated in phase I clinical trial in the case of head and neck squamous cell carcinoma (Riechelmann et al. 2008). Targeting CD44 by an A6 peptide (acetyl-KPSSPPEE-amino) blocked the migration and metastasis of CD44-expressing cells

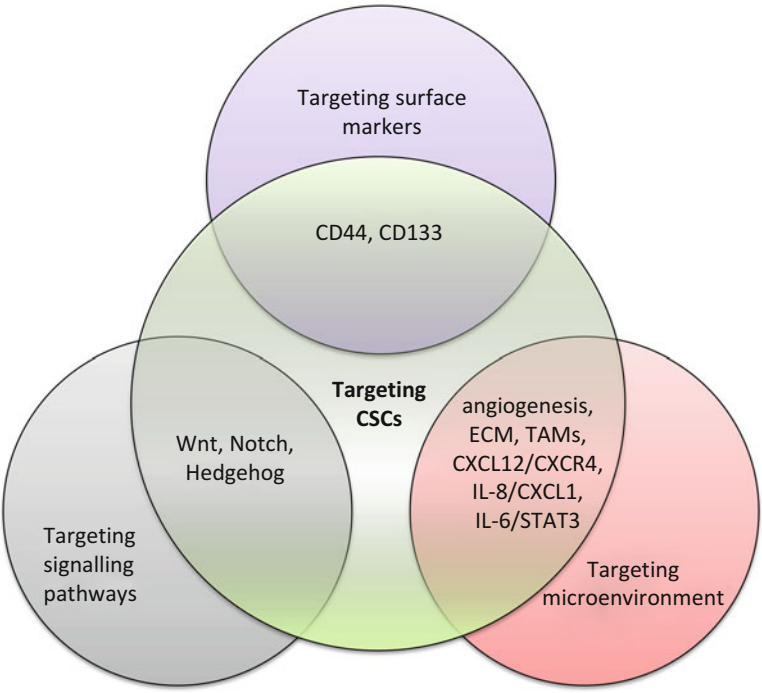

Fig. 2.4 Therapeutic targets potentially useful in anti-CSCs therapy. *ECM* extracellular matrix, *TAMs* tumor-associated macrophages

(Piotrowicz et al. 2011). In hepatocellular carcinoma, neutralizing CD44 can also inhibit $CD90^+$ cell-mediated tumor formation and metastasis in vivo, suggesting an therapeutic strategy against $CD90^+$ CSCs by targeting CD44 (Lee et al. 2013).

In an acute myeloid leukemia mouse model, in vivo administration of an activating monoclonal antibody directed at CD44 resulted in significant reduction in the levels of leukemic repopulation (Jin et al. 2006). CD44 is a regulator of several miRNAs known to maintain CSCs. When CD44 expression in PCa cells is downregulated, miR-34a levels increase leading to reduced tumor regeneration and metastasis in xenografts (Liu et al. 2011a).

7.2 Targeting Self-Renewal and Differentiation Pathways

Signaling pathways, such as Wnt, Notch, and Hedgehog (Hh), are essential for both regulation of EMT/metastasis and self-renewal of CSCs in several cancers. Development of agents that target critical steps in these pathways will be complicated due to signaling cross-talk (Takebe et al. 2011). Several novel agents targeting Wnt/β-catenin have been developed. Some of these agents have been shown to

selectively target the cancer stem cell subpopulation in vivo, inhibit tumor growth and inhibit metastasis (Takebe et al. 2011). Inhibition of Notch1 can significantly decrease the $CD44^{+}CD24^{-}$/low subpopulation and inhibited the development of brain metastases from breast cancer (McGowan et al. 2011). Pharmacologic blockage of aberrant Hedgehog signaling might be an effective therapeutic strategy for inhibiting metastases in human cancers through targeting CSCs. A small-molecule Hedgehog inhibitor, IPI-269609, has been proved to profoundly inhibit systemic metastases in orthotopic xenografts derived from human pancreatic cancer cell lines, accompanied with a significant reduction in the population of ALDH-positive cells in pancreatic cancer (Feldmann et al. 2008).

Salinomycin, a wnt/β-catenin inhibitor, inhibits tumor growth, induces epithelial differentiation of tumor cells, and down-regulates CSC genes in tumor cells (Gupta et al. 2009).

As stem cells are often dependent on bone morphogenetic protein (BMP) signaling, a number of therapeutic strategies are being sought to target this pathway (Joseph et al. 2012; Zhang et al. 2003; Zhu and Emerson 2004). Piccirillo et al. (2006) reported that the BMP-BMPR signaling system – which controls the activity of normal brain stem cells – may also act as a key inhibitory regulator of tumor-initiating, stem-like cells from glioblastoma by a reduction in proliferation and increased expression of markers of neural differentiation. The reduction in the size of the $CD133^{+}$ population and the growth kinetics of the glioblastoma cells suggest that targeted BMP-pathway therapeutics are worth pursuing (Massard et al. 2006; Piccirillo et al. 2006).

The histone deacetylase inhibitor valproic acid, commonly used to treat epilepsy, has recently been found to have anti-cancer activity that may target CSCs (Blaheta et al. 2005). Valproic acid induces the terminal differentiation of cancer cells by increasing the DNA binding of activating protein-1 transcription factor, decreasing protein kinase C (PKC) activity, inhibiting the Wnt signaling pathways, and activating the peroxisome proliferator-activated receptors, in addition to blocking histone deacetylase (Blaheta et al. 2005).

Histone deacetylase (HDAC) inhibitors inhibit growth, induce differentiation and apoptosis of neurosphere derived from glioblastoma (GBM). GBM neurospheres contain cancer stem cell like that propagate tumor and resist cytotoxic therapeutics. Using MS-275, a specific gene product induced by HDAC inhibition, Delta/Notch like epidermal growth factor (DNER), inhibited the growth of GBM derived neurospheres, induced their differentiation and inhibited their engraftment and growth as tumor xenografts (Sun et al. 2009). The HDAC inhibitor entinostat reverses EMT in xenografts (Tate et al. 2012).

7.3 Differentiation Therapies

Another proposed therapeutic approach is to stimulate differentiation of CSCs such that they lose their capacity for self-renewal and resistance to chemotherapeutic agents (Nguyen et al. 2012). This is particularly critical where CSCs are widely

distributed at low density, making conventional interventions challenging. Thus far, the most well developed therapeutic agent is vitamin A and its analogues (retinoid acid) for the treatment of acute promyelocytic leukemia (APL). These agents enhance tumor differentiation and reverse malignant progression by modulation of signal transduction networks regulated by nuclear retinoid receptors. In patients with APL, a 90 % remission rate and a 70 % cure rate with all-trans retinoic acid therapy followed by chemotherapy has been observed (Burnett et al. 2010). In vitro, retinoid acid can also induce differentiation in embryonic cells, teratocarcinomas and melanomas (Rohwedel et al. 1999).

7.4 Therapies Directed at CSC Niche and Pre-metastatic Niche

The challenges of targeting disseminated CSCs may be even more pronounced, as the distant microenvironment may help protect these cells from therapeutic insults (Hovinga et al. 2010).

7.4.1 Targeting Vasculature

In particular, the vasculature likely plays an important role in forming stem and progenitor cell niches and has been suggested to regulate many tumor microenvironments (Bautch 2011). Therefore, damaging the CSC niche environment may impact the survival and tumor-initiating properties of CSCs (Folkins et al. 2007). The impact of angiogenesis inhibitors such as bevacizumab, thalidomide, sorafenib, sunitinib, pazopanib may be in part related to their effects on the vascular niche and disruption of the CSC microenvironment (Tonini et al. 2003).

The VEGF-specific antibody bevacizumab has direct and rapid anti-vascular effects and seem to be useful in targeting CSCs by disturbing niche (Willett et al. 2004). On the other hand, hypoxic tumor microenvironment promotes tumor progression, regulates CSCs and increases their metastatic potential (Hill et al. 2009). Inhibition of hypoxia eliminates metastasis in mice without effect on the primary tumor, suggesting that hypoxia is an important process in the formation of pre-metastatic niche (Sceneay et al. 2013).

Preclinical models suggest that antiangiogenic agents actually increase invasive and metastatic properties of breast cancer cells (Ebos et al. 2009; Pàez-Ribes et al. 2009). Hypoxia induced by administration of antiangiogenic agents might accelerate tumor growth and metastasis by increasing the CSC population. Conley et al. (2012) demonstrated that administration of sunitinib and bevacizumab increased CSC population in breast cancer xenografts as a consequence of tumor hypoxia, and this effect was mediated by HIF-1α through the activation of Wnt pathway via Akt/β-catenin signalling. Authors concluded that antiangiogenic agents might have to be combined with CSC targeting drugs.

These results differ from those obtained in glioblastoma. Glioblastomas express high levels of vascular endothelial growth factor (VEGF) (Bao et al. 2006b). A functional interaction between brain CSCs and endothelial cells is supported by the close association of $CD133^+$ brain cancer cells with vascular endothelial cells in vitro and in vivo, and more importantly by the demonstration that coinjection of primary human endothelial cells enhances tumor formation by $CD133^+$ medulloblastoma cells in immune-deficient mice (Calabrese et al. 2007). Tumors initiated in mice by $CD133^+$ cells from either primary glioblastoma biopsy specimens or xenograft cell lines are highly vascular (Bao et al. 2006b) Treatment of xenograft tumors with bevacizumab not only potently inhibits tumor growth in mice (Calabrese et al. 2007; Bao et al. 2006b) but also results in depletion of cells coexpressing CD133 and nestin, a marker of primitive neural cells, without directly affecting bulk tumor cell proliferation or death. Together, these results suggest that inhibition of brain tumor growth by antiangiogenic agents is mediated at least in part by disruption of a vascular niche required for maintenance of CSCs.

7.4.2 Targeting the Extrinsic Signals at the CSC Niche

The CXCL12/CXCR4 plays a central role in cancer cell proliferation, invasion, and dissemination in the majority of malignant diseases. Although the signals generated by the metastatic niche that regulate CSCs are not yet fully understood, accumulating evidence suggests a key role of the CXCL12/CXCR4 axis (Cojoc et al. 2013). Strategies aimed at modulating the CXCL12/CXCR4 axis may have important clinical applications to inhibit CSC growth (Gil et al. 2014; Barone et al. 2014). In a phase I study evaluating LY2510925, a peptide agonist blocking stromal cell derived factor-1 (SDF1) from CXCR4 binding, in 45 advanced cancer patients, stable disease was obtained in nine (20 %) of them (Galsky et al. 2014).

Multiple agents are currently being developed to target CXCL12/CXCR4 signaling in cancer. The anti-CXCR4 drug AMD3100 (plerixafor) is approved for stem cell mobilization in patients with non-Hodgkin's lymphoma and multiple myeloma; the CXCL12 analog CTCE-9908 is approved for clinical use in patients with osteosarcoma. Novel CXCR4 antagonists are currently in clinical trials for multiple myeloma, leukemia, and lymphoma. CXCR4 inhibitor MSX-122 is in Phase I trials for advanced malignant disease resistant to standard therapy. NOX-A12 neutralizes CXCL12 and is in clinical trial for the treatment of chronic lymphocytic leukemia and multiple myeloma (Ramsey and McAlpine 2013; De Nigris et al. 2012). AMD3100 has been shown to decrease metastatic potential in animal models for different types of tumors (Smith et al. 2004; Kim et al. 2010; D'Alterio et al. 2012; Kajiyama et al. 2008; Matsusue et al. 2009). Similarly, blocking CXCR4 receptor function by a monoclonal antibody or polypeptide inhibits cancer cell proliferation, motility, and invasion in multiple preclinical models both in vitro and in vivo (Zeng et al. 2006; O'Boyle et al. 2013). Recent data suggest that inactivation of the CXCL12/CXCR4 axis by neutralizing antibody or by the CXCR4-specific small

molecule antagonist AMD3100 inhibits glioma, renal, colon, pancreas, and prostate cancer progenitors as well as tumor initiating population within gefitinib-resistant lung cancer and tamoxifen-resistant breast cancer cells in vitro and in animal models (Gassenmaier et al. 2013; Dubrovska et al. 2012; Redjal et al. 2006). Low oxygen tension is a critical microenvironmental factor in regulating tumor initiating axis in cancer cells; hypoxia promotes expansion of CSCs and converts non-stem cancer cells into CSC populations with increased self-renewal capacity (Heddleston et al. 2009; Soeda et al. 2009). The effects of reduced oxygen tension on CSCs are mediated at least in part through the activation of the HIF signaling pathway (Li and Rich 2010). CXCR4 expression is induced under hypoxic stress via activation of the HIF pathway (Ishikawa et al. 2009). As tumor cells can be protected from the effect of ionizing radiation by hypoxia, pharmacologic inhibition of the CXCL12/CXCR4 interaction by AMD3100 or neutralizing antibody prevents the recurrence of glioblastoma after irradiation in mice by inhibition of vasculogenesis (Kioi et al. 2010). Activation of CXCR4-mediated STAT3 signaling in non-small cell lung cancer cells is functionally crucial for the maintenance of stemness and resistance to radiotherapy (Jung et al. 2013). Recent prostate tumor xenograft studies in mice showed that a combination of AMD3100, which targets prostate cancer stem-like cells, and the conventional chemotherapeutic drug docetaxel, which targets the bulk tumor, is significantly more effective in eradicating tumors as compared to monotherapy (Dubrovska et al. 2010, 2012; Domanska et al. 2012).

Human breast cancer cells expressing CXCR1, a receptor that binds the proinflammatory chemokine IL-8, are present almost exclusively within the CSC-containing ALDH1$^+$ population (Ginestier et al. 2010). IL-8 has been implicated in tumor invasion, metastasis, and self-renewal. Treatment of orthotopically transplanted tumors in NOD/SCID mice with the CXCR1/2 inhibitor repertaxin, the standard chemotherapeutic agent docetaxel, or a combination of both drugs all resulted in impaired tumor growth. However, tumors treated with docetaxel alone showed either unchanged or increased percentage of ALDH1$^+$ cells compared with untreated controls, whereas repertaxin treatment alone or in combination with docetaxel significantly reduced the ALDH1$^+$ population. Upon serial transplantation, tumor cells derived from control or docetaxel-treated primary animals were able to generate tumors in secondary mice with similar efficiency, while cells from repertaxin-treated animals showed a two- to fivefold reduction in tumor growth and were only able to generate tumors at the highest injected cell dose (Ginestier et al. 2010).

STAT3 signalling has an important role in self-renewal and differentiation of stem cells. IL-6 is implicated in promoting STAT3 mediated CSC expansion in several other types of tumors. Expression levels of IL-6 and its receptor are highly elevated in prostate CSCs, and a crucial role of JAK-STAT3 in mediating IL-6-induced stem cell maintenance in prostate cancer has been shown. Furthermore, IL-6–JAK2–STAT3 signalling is required for the maintenance of breast CSCs and tumor growth (Yu et al. 2014). In addition, IL-6 induces the recruitment of mesenchymal stem cells (MSCs), into the tumor microenvironment. IL-6 increases STAT3

activation in MSCs, which contributes to MSC survival and MSC-mediated tumor progression (Rattigan et al. 2010).

Given the central role of STAT3 in the promotion and maintenance of a stem cell phenotype, controlling STAT3 activity in this population should inhibit tumor progression. In stem cells, STAT3 activity can be regulated by EZH2-mediated protein methylation (Kim et al. 2013). STAT3 protein phosphorylation affects the regulation of genes driving stemness, EZH2-induced STAT3 protein methylation, and possibly also STAT3 acetylation induced by p300 acetyltransferase, and is thus crucial for regulating the formation of a transcription complex bound to the promoters of genes with a propensity to promote stem cell characteristics. Therefore, functional disruption of STAT3 modification enzymes such as EZH2 and p300 may serve as a promising therapeutic strategy for human cancers. However, targeting these modification enzymes may generate broad biological effects that lead to unwanted toxicity (Yu et al. 2014).

7.4.3 Targeting Tumor Associated Macrophages

Tumor associated macrophages (TAMs) play an important role in tumor growth, angiogenesis, metastasis, matrix remodelling and immune evasion in various human and animal tumors (Sica and Bronte 2007; Biswas et al. 2008; Solinas et al. 2009; Sica et al. 2008). In mouse tumor models, an increased density of TAMs is associated with poor efficacy of chemotherapy and radiotherapy (Zhang et al. 2010; Meng et al. 2010). The density, activation and histological location of TAMs can predict patients' survival in different types of cancer (Zhu et al. 2008; Kurahara et al. 2011; Hanada et al. 2000). Therefore, TAMs are now considered as a promising target for tumor therapy. Some tumor-released and stroma-released cytokines and chemokines facilitate the recruitment of macrophages to tumor tissues, and possible therapeutic strategies are aimed at inhibiting macrophage recruitment. For example, overexpression of C-C motif chemokine ligand 2 (CCL2) was correlated with macrophage infiltration and poor prognosis in human cancers (Roca et al. 2009); macrophage infiltration and the growth of tumors were reduced when CCL2 was inhibited (Mizutani et al. 2009; Qian et al. 2011; Zhu et al. 2010). A CCL2-targeting agent, trabectedin, used in clinic to treat human ovarian cancer and myxoid liposarcoma, could suppress the recruitment of monocytes to tumor sites and inhibit their differentiation to mature TAMs (Allavena et al. 2005; Germano et al. 2010).

In a phase II clinical study, siltuximab, anti-interleukin- 6 (IL-6) antibody, reduced macrophage infiltration in tumor tissue by decreasing the plasma level of some chemoattractants such as CCL2, vascular endothelial growth factor (VEGF) and C-X-C motif chemokine ligand-12 (CXCL-12) (Coward et al. 2011). An alternative way to suppress the chemoattractive activity of CCL2 is neutralizing its receptor, C-C motif chemokine receptor 2 (CCR2). A CCR2 inhibitor, RS102895, has exhibited negative effects on macrophage migration (Jin et al. 2010). Another important chemoattractant for macrophages is macrophage colony-stimulating factor (M-CSF). In human hepatocellular carcinoma, there is a significant association

between high M-CSF expression and high macrophage density, each relates to poor overall survival of patients (Zhu et al. 2008). Treatment with M-CSF antibody suppressed tumor growth by 40 % in human MCF-7 breast cancer xenografts (Paulus et al. 2006). Two M-CSF receptor inhibitors (JNJ-28312141 andGW2580) were found to decrease TAM count and suppress tumor growth, angiogenesis and metastasis (Manthey et al. 2009; Kubota et al. 2009).

Other chemoattractants for macrophages, such as VEGF, CXCL-12 and CCL5, also seem to be potential targets for TAM depletion. Selectively inhibiting VEGFR-2 reduced macrophage density and prevented tumor growth and angiogenesis in orthotropic pancreatic and breast tumors (Dineen et al. 2008; Roland et al. 2009). Repressing either the CXCL12/CXCR4 or the placental growth factor (PIGF)/VEGFR-1 pathway reduced macrophage count (Welford et al. 2011). The tumor microenvironment is usually hypoxic and hypoxia-inducible factors are transcriptional activators for VEGF and CXCR4 genes (Fang et al. 2009); HIF-1a deficiency reduced macrophage density, tumor angiogenesis and invasion in murine glioblastoma via blocking the matrix metalloproteinase 9 (MMP9)/VEGF pathway (Du et al. 2008). HIF-2a mediates macrophage migration to the tumor microenvironment partly through regulating M-CSFR and CXCR4 (Imtiyaz et al. 2010).

Some drugs commonly used in clinical practice can directly suppress TAMs survival. Clodronate has a selective cytotoxicity to macrophages and this clodronate-induced depletion of macrophages can result in the regression of tumor growth, angiogenesis and metastasis (Zeisberger et al. 2006; Hiraoka et al. 2008). Zoledronic acid selectively depletes MMP9-expressing TAMs (Tsagozis et al. 2008), impairs the differentiation of myeloid cells to TAMs and induces the tumoricidal activity of macrophages (Tsagozis et al. 2008; Veltman et al. 2010; Coscia et al. 2010). Dasatinib, a Src kinase inhibitor and a preclinical drug for chronic-phase chronic myeloid leukemia, could reduce $MMP9^{+}$ macrophage density and inhibit MMP9 expression in the tumor microenvironment (Liang et al. 2010).

Another approach is to deplete TAMs by targeting their surface molecules with immunotoxin-conjugated agents. In ovarian cancer, alemtuzumab (anti CD52) induced lysis of myeloid cells in vitro and ex vivo, supporting the use of lemtuzumab in clinical trials to test its efficacy as an anti-myeloid cell antiangiogenic therapeutic (Pulaski et al. 2009). Folate receptor b (FRb) is another surface protein over-expressed in M2-like TAMs (Nagai et al. 2009; Puig-Kröger et al. 2009), and the existence of FRb^{+} macrophages positively associates with high vessel density, high incidence of haematogenous metastasis and a poor prognosis in patients with pancreatic cancer (Kurahara et al. 2012). A recombinant immunotoxin to folate receptor beta affects tumor growth, accomplished with the depletion of TAMs (Nagai et al. 2009). In this approach while pro-tumoral M2 TAMs could be depleted, the M1 tumoricidal ones are not affected.

Inhibiting the signals essential for M2 differentiation so impairing the pro-tumoral and immunosuppressive profile of TAMs is another strategy in development. STAT3 pathway is consistently active in many tumors and acts as a negative regulator for macrophage activation and the host's inflammatory responses. When the activation of STAT3 was blocked, either with a dominant negative variant or an

antisense oligonucleotide, macrophages could increase the release of IL-12 and RANTES and reverse the systemic immune tolerance (Cheng et al. 2003). Two tyrosine kinase inhibitors (sunitinib and sorafenib) have shown their inhibitory effects on STAT3 in macrophages in vitro (Xin et al. 2009; Edwards and Emens 2010). Sorafenib can restore IL-12 production but suppress IL-10 expression in prostaglandin E2 conditioned macrophages, indicating its effects on reversing the immunosuppressive cytokine profile of TAMs (Edwards and Emens 2010). Another STAT family member important for TAM biology is STAT6. In one study, $STAT6^{-/-}$ mice produced predominantly M1-like tumoricidal TAMs and >60 % of $STAT6^{-/-}$ mice rejected tumor metastasis (Sinha et al. 2005). Several up-/down-stream mediators of STAT6 could act as modulators of TAM function. These modulators include phosphatidylinositol 3-kinase (PI3K), Src homology 2-containing inositol-5′-phosphatase (SHIP), Kruppel-like factor 4 (KLF4) and c-Myc.

It has been reported that the expression of KLF4 was induced in M2 macrophages and reduced in M1 macrophages. A study indicated that KLF4 cooperated with STAT6 to induce an M2 pattern. Levels of KLF4 can be manipulated by diverse agonists such as statins, resveratrol, bortezomib and dietary compounds (Liao et al. 2011). Other proteins and signalling pathways are known to promote M2-like properties of macrophages and are also the potential targets for tumor therapy. Peroxisome proliferator-activated receptor (PPAR)-c can promote M2 type differentiation of human macrophages by acting as a transcriptional inhibitor of NF-jB.132 PPAR-a plays a role in macrophages by antagonizing M1 polarization and supporting M2 polarization (Van Ginderachter et al. 2008). As synthetic inhibitors of PPAR-a/c have now been identified, the evaluation of their role in TAM targeted therapy is essential.

HIFs are a possible target because of their over-expression in TAMs residing in the hypoxic tumor microenvironment and their ability to induce the production of angiogenic factors, including VEGF, platelet-derived growth factor-b, NOS2, fibroblast growth factor 2, IL-8 and cyclooxygenase-2. Macrophage-targeted depletion of HIF-1a reduced tumor growth in mice (Doedens et al. 2010).

Among anti-tumor drugs, cisplatin promotes macrophages to produce large amounts of NO, a reactive oxygen intermediate and proinflammatory cytokines, leading to enhanced tumoricidal activity (Chauhan et al. 2009). Silibinin inhibits the production of angiogenic cytokines and interleukins in macrophages, leading to angiogenesis regression (Tyagi et al. 2009). Finally, pantoprazole enhances TAM recruitment but increases TAMs to an M1-like tumoricidal state (Vishvakarma and Singh 2010).

8 Future Prospects

Metastatic cancer remains an incurable disease and targeting CSCs is a novel promising approach. Many drugs active on CSCs or metastatic niche are already used in clinical practice; other approaches are under clinical trials or still in a preclinical

phase (Ferrari and Nicolini 2012; Ferrari et al. 2013). It is likely that drugs targeting CSCs or their microenvironment would be mostly useful in an early phase of cancer history, when there is a micrometastatic spread not clinically evident. In this context, such drugs, eventually combined with standard anticancer drugs, could theoretically eradicate the minimal residual disease.

Targeting only CSCs may not be enough to prevent metastasis or relapse due to metastasis. Continued development of combination therapies with multiple targets (e.g. targeting CSCs, combination of chemotherapy, differentiation therapy, and targeting microenvironment) will be essential. A study demonstrated that a neutralizing antibody against a membrane-associated NOTCH ligand inhibits tumor growth and CSC self-renewal in human colon cancer implanted mice (Hoey et al. 2009). Another study showed that the administration of an anti-CD123 antibody prevents the engraftment of serially transplanted acute myeloid leukemia into the animals, suggesting this antibody impedes the stem-like characteristics of leukemia cells (Jin et al. 2009). In both cases, the inhibitory abilities of the antibodies were enhanced in combination with chemotherapy. Releasing CSCs from their niche could enhance their susceptibility to chemotherapy. Indeed, when acute myeloid leukemia cells (Nervi et al. 2009) and multiple myeloma cells (Azab et al. 2009) are treated prior to chemotherapy, with a CXCR4 inhibitor, that prevents the lodging of cancer cells into select microenvironments, the chemosensitivity of these cells was strongly enhanced. In part, release from the protection of the microenvironmental niche could sensitize CSCs to chemotherapeutics. It is also possible that disruption of CXCL12/CXCR4 signaling activates CSC cycling which in turn could sensitize CSCs to agents targeting proliferating cells. This strategy could be helpful for targeting potentially dormant DTCs in patients with no clinically apparent distant disease (Shiozawa et al. 2013).

Tumor dormancy is another important issue. In fact, as most neoplasms are identified after they have reached a critical mass, the ability to block the reactivation of dormant CSCs at distant sites of metastases is a critical area of research. Dormancy of disseminated tumor cells (DTCs) may not be a process exclusive to metastatic cells that arise from established primary tumors. In fact, pre-invasive lesions also contain epithelial cells that can undergo epithelial–mesenchymal transition and disseminate; these cells are referred to as early DTCs. Early DTCs can develop metastatic growth capacity that manifests after long periods of dormancy. By disseminating at early stages, DTCs that survive may evolve divergently from the primary tumor. This may generate metastases with different characteristics from the primary lesion and may explain the lack of success of treating metastasis with therapies designed on the basis of primary tumor characteristics. Moreover, the vast majority of early DTCs in mouse models seem to be dormant, and clinical evidence supports this hypothesis. This suggests that persistence in a dormant state may protect these DTCs from treatment, contributing to late recurrence of disease (Sosa et al. 2014). Possible therapeutic strategies mimic the dormancy programme to sustain dormant DTCs and thereby prevent relapse. Cancer therapy may force surviving residual tumor cells into dormancy by activating stress signalling (Schewe and Aguirre-Ghiso 2009; Kobayashi et al. 2011). An example of this may be tumor cells that are known as drug-tolerant

persisters that survive targeted therapies by altering epigenetic mechanisms (Sharma et al. 2010). Drugs commonly used in clinic may be useful to induce dormancy in DTCs. One study showed that, in both primary cells and breast cancer and leukemia cell lines, the DNA methylation inhibitor 5-azacytidine alone caused decreased expression of G0 to G1 exit genes (Tsai et al. 2012). Also, HDAC inhibitors or DNA demethylating agents might represent alternative adjuvant therapies to induce prolonged dormancy of uveal melanoma or other types of DTCs (Sosa et al. 2014).

Among new therapeutic targets, ECM has gained importance in the recent years. Recent advances in knowledge about the role of TNC and POSTN in the metastatic microenvironment suggest that these ECM components as new therapeutic targets (Malanchi et al. 2011; Oskarsson et al. 2011).

As pro-tumoral activity of TAMs largely depends on their recruitment and activation, therefore therapeutic strategies against TAMs should be aimed at inhibiting macrophage recruitment, suppressing TAM survival, enhancing M1 tumoricidal activity of TAMs and blocking M2 tumor-promoting activity of TAMs. So far, many agents have been identified as candidate drugs, either as inhibitors of macrophage accumulation or as modulators of TAM properties. Using immune system to treat cancer is a promising approach. As TAMs contribute to chemoresistance and radioprotective effects, TAM-targeted strategies may also improve the efficacy of conventional therapies in some cases (Tang et al. 2013).

Finally, an important step will be the identification of additional markers that provide even more specific isolation and characterization of CSCs, particularly in solid tissues, particularly markers that can be used for localization and visualization of CSCs in situ, to facilitate anatomical localization of the niche as well. However, it will likely be a significant task given the complexity of the niche, comprising fibroblastic cells, myeloid and other inflammatory cells, endothelial and perivascular cells (or their progenitors), and ECM components (Sneddon 2007). Functional studies will be crucial for understanding the contribution of defined molecular constituents of metastatic niche to CSC physiology.

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Chapter 3
Cytokine Networks and Cancer Stem Cells

Clifford Liongue, Alister C. Ward, Wei Duan and Sarah Shigdar

Abstract Cell-to-cell communication is an integral function of multicellular organisms. Many of these signals are received by a myriad of cell-surface receptors that utilize a range of intracellular signaling pathways to communicate this to the nucleus, rapidly impacting on the transcription of target genes in order to elicit the desired response, such as proliferation, differentiation, activation, and survival. Dysregulation of these important signaling pathways, and networks, often lead to pathological conditions due to inappropriate cell responses with negative consequences. The aberrant signaling pathways have been associated with many diseases, including cancer. Cytokines and chemokines convey a multitude of messages to the target cell, many of which are beneficial for cancers and cancer stem cells, such as proliferation, survival and migration. By hijacking this communication network, cancers and cancer stem cells can become invasive and more pathogenic. Furthermore, by using these communication systems, cancer stem cells are able to evade current therapies. Therefore, novel therapies may be developed to break the communication systems of the cancer stem cells. This chapter explores the role of the cytokines TGF-β, TNF-α, IL-1 and IL-6 and chemokine CXCL8 as well as NF-κB and their role in cancer stem cell survival and maintenance. Emerging therapies are beginning to target the cancer stem cell population, either specifically or synergistically with existing therapeutic options. These novel therapies may hold the key to breaking the communication network of cancer stem cells.

Keywords Cancer • Cancer stem cells • Cytokines • Inflammation • Signaling networks

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1 Introduction

During development and throughout life, cells constantly respond to external stimuli and generate an appropriate response, such as proliferation, differentiation, activation and survival. Among these stimuli are cytokines and chemokines, which play an essential role in the development and regulation of a range of cell types, in particular those of the immune and hematopoietic systems. Cytokine signaling plays a major role in the initiation, propagation and resolution of inflammation. Given that cancer is now recognized as a disease of inflammation, it is no surprise that cytokines contribute strongly to the development and propagation of cancer and cancer stem cells. This Chapter aims to explore the contribution of cytokines to the phenotype of cancer stem cell phenotype, as well as identifying possible therapeutic targets and postulating on the effects that personalized medicine may have on the future treatment of cancer patients.

2 Tumor Microenvironment, Inflammation, EMT and Cancer Stem Cells

Inflammation is a natural response to injury (Coussens and Werb 2002). The presence of a tumor triggers an inflammatory response, creating a microenvironment that may enhance the dissemination of cancer cells (Hanahan and Weinberg 2000, 2011). Key features of the tumor microenvironment include endothelial cells, bone marrow derived stromal cells and infiltrating white blood cells, which migrate into the tumor following cytokine and chemokine recruitment via the tumor cells or surrounding stroma (Grivennikov et al. 2010; Hanahan and Weinberg 2011). Within the tumor mass are so-called cancer stem cells (CSCs), which are a small subset of cells with the ability to proliferate and form new tumors (Al-Hajj et al. 2003; Koch et al. 2010). The key functional properties of these cells are self-renewal, multi-potent differentiation and the capacity to generate a heterogeneous lineage of all types of cancer cells comprising a tumor (Shackleton 2010; Clevers 2011). These CSCs also have the potential to lose their epithelial phenotype, becoming motile and invasive, and taking on a mesenchymal phenotype, a process known as the epithelial-to-mesenchymal transformation (EMT), and representing an important step in metastasis (Singh and Settleman 2010; Biddle and Mackenzie 2012; Brabletz 2012).

A number of cytokines and chemokines have been shown to be released during inflammation that play a vital role in the progression of cancer, affecting key CSC phenotypes such as EMT. These include Interleukin (IL)-1, IL-6, IL-8 (CXCL8), tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-β) and NF-κB. Communication between cells is not unidirectional, with both immune and tumors cells producing cytokines and chemokines (Lu et al. 2006). Such cues, as along with additional environmental factors, induce the changes that enable cancer cells to metastasize (Hanahan and Weinberg 2011).

Tumor associated macrophages (TAMs) play a major role in the progression of cancer. The secretion of IL-1 and TNF-α by TAMs has been shown to support the steps involved in invasion and metastasis (Biswas et al. 2013). Secretion of TNF-α by TAMs and other immune cells can also up-regulate the secretion of TGF-β by cancer associated fibroblasts (CAFs) and immune cells, which is an inducer of IL-1, IL-6 and IL-8 (Mani et al. 2008). IL-6 is also secreted by CAFs, TAMs, other immune cells, as well as by the cancer cells themselves, forming a positive feedback loop (Shigdar et al. 2014). The cancer cells also secrete IL-8, MCP-1 and RANTES, which promote proliferation of stromal cells, including endothelial cells (Levina et al. 2008), with RANTES and MCP-1 also able to attract tumor-infiltrating lymphocytes (Ji and Zhang 2010). Both IL-6 and IL-8 have been shown to mediate chronic inflammation, suggesting that cancer cells are directly involved in stimulating the inflammatory process (Korkaya et al. 2011, 2012).

How then do these cytokines and the inflammatory process contribute towards a CSC phenotype? This ultimately relates to the downstream signaling pathways induced, which act on key genes to modify the phenotype of CSCs. However, this is a multi-step process. Using the analogy of the 'never-healing wound', proliferating tumor cells and the surrounding activated stroma secrete cytokines and chemokines to attract immune cells which secrete further factors to induce activation and/or infiltration of other cells. The center of this 'ball of cells' becomes hypoxic, leading to the up-regulation of Hypoxia-inducible factor-1 alpha (HIF-1α), which acts in concert with cytokine-induced Snail, Twist1 and Stat3 to induce epithelial-mesenchymal transition (EMT). This then leads to the dissemination of CSCs to distant metastatic sites, where these cells then initiate secondary tumors (Mani et al. 2008), but also leaving behind a population of CSC-like cells (Singh and Settleman 2010; Kong et al. 2011). Indeed, it is thought that the de-differentiation of cancer cells into CSCs, is the first step in the process towards EMT (Fig. 3.1). Moreover, the aberrant activation of EMT enhances cancer cell motility and dissemination but also confers a stem-cell like phenotype, as evidenced by gene expression patterns, and leads to an increase in the CSC population (Chaffer et al. 2011; Hanahan and Weinberg 2011; Brabletz 2012), pointing to the close association between CSCs and EMT. However, cytokines are also important in maintaining CSCs and inducing other phenotypes in these cells in addition to EMT.

3 Transforming Growth Factor-Beta

Transforming growth factor-beta (TGF-β) is secreted by immune cells during normal wound healing in order to trigger surrounding epithelial cells to undergo EMT enabling them to migrate to the site of the wound for repair (Biddle and Mackenzie 2012) (Fig. 3.2). However, this process becomes perturbed during cancer development. Immune cells migrate to the area of a tumor in an attempt to heal the 'wound' and secrete cytokines, such as TGF-β. The cancer cells, meanwhile, have developed oncogenic mutations that render them more responsive to these

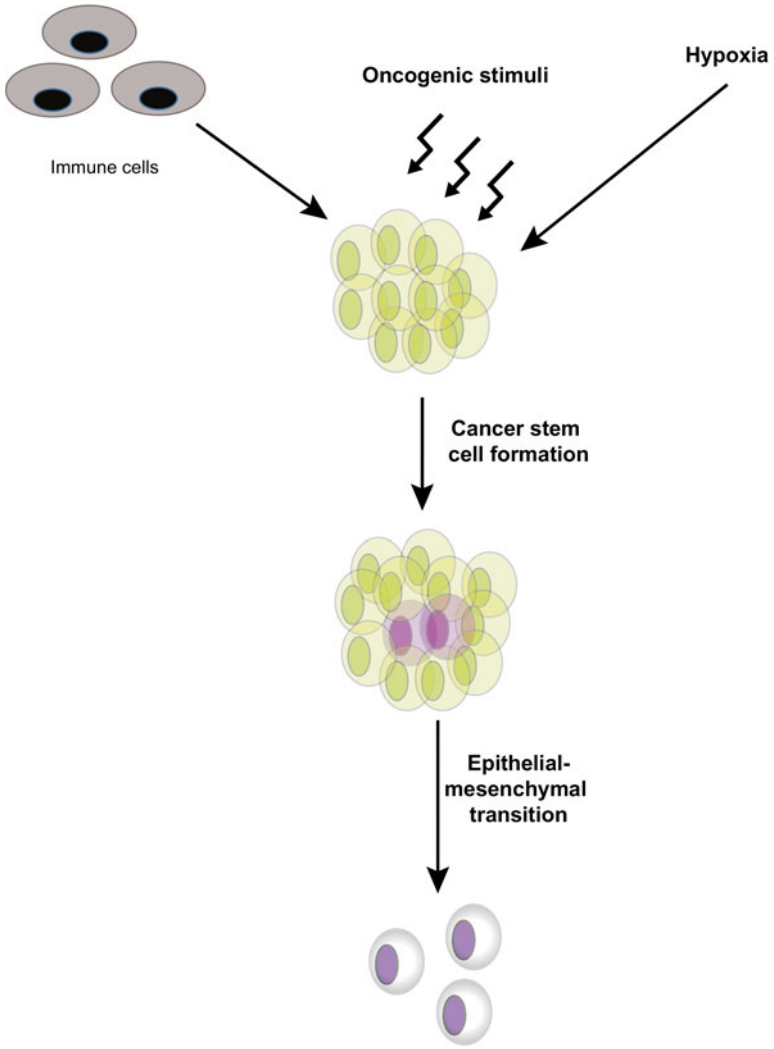

Fig. 3.1 *Factors promoting cancer stem cell formation*. Schematic representation of the link between tumor microenvironment, inflammation, and oncogenic signaling in cancer stem cell formation. The CSCs develop a number of phenotypes, including migratory capacity via an epithelial-mesenchymal transition enabling the dissemination of the tumor (Adapted from (Mani et al. 2008; Singh and Settleman 2010; Kong et al. 2011; Shigdar et al. 2014))

EMT-inducing signals (Biddle and Mackenzie 2012), while also converting TGF-β's normal growth inhibitory role into a growth promoting role (Padua and Massague 2009; Asiedu et al. 2011). To understand how TGF-β induces EMT, and thus CSCs, an understanding of the signaling pathways involved is required to induce EMT, TGF-β signals via both a Smad-dependent and Smad-independent transcriptional pathway (Fig. 3.3). The Smad-dependent pathway involves phosphorylation of

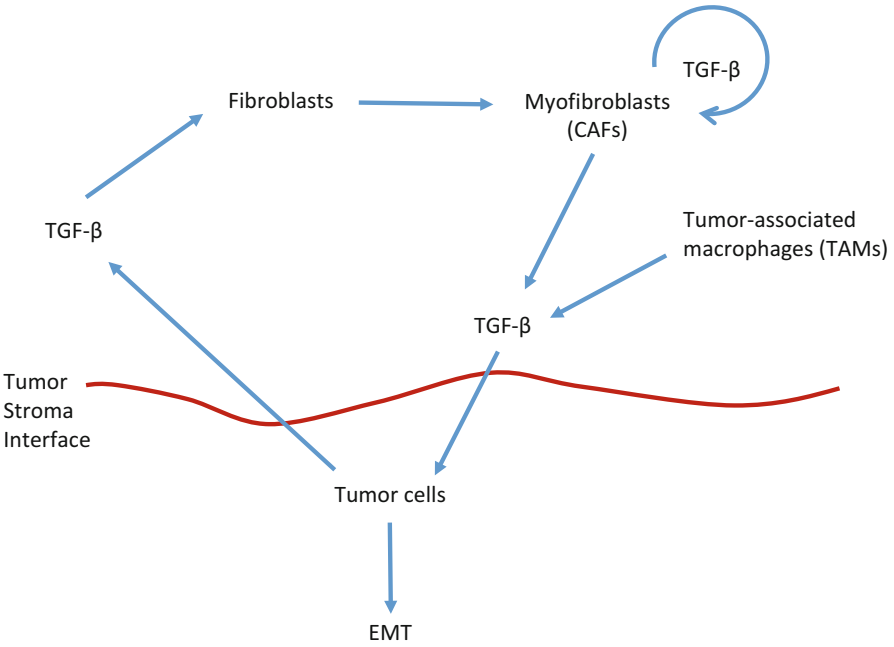

Fig. 3.2 *Role of TGF-β in CSC phenotypes.* Mode of action of paracrine and autocrine TGF-β signaling involved in cancer stem cell phenotypes, including epithelial-mesenchymal transition (Adapted from (Biddle and Mackenzie 2012))

Smad2 and Smad3, which then form heteromeric complexes with Smad4. These complexes then translocate into the nucleus where they control transcription of EMT target genes (Massague 2000; Derynck and Zhang 2003). The Smad-independent pathway activates Ras/Erk, c-Jun N-terminal kinase (JNK), phosphatidylinsitol-3 (PI3) kinase, Par6, and Cdc42 GTPases, which have also been associated with TGF-β-induced EMT (Derynck and Zhang 2003).

Autocrine TGF-β signaling has been linked to EMT and migration, as well as maintenance of a stem cell-like population. Indeed, TGF-β secreted by cancer cells can mediate the differentiation of fibroblasts into myofibroblasts, or CAFs, which then secrete more TGF-β into the microenvironment, thus further promoting EMT (Fig. 3.2). In a recent study, the abrogation of TGF-β autocrine signaling resulted in decreased expression of vimentin and Snail, and enhanced expression of E-cadherin, indicating a reduction in EMT potential. In addition, the cancer stem cell-like markers Integrin β1, Notch 1 and aldehyde dehydrogenase 1/2 were also reduced. Finally, expression of Gli1, a component of the hedgehog signaling pathway involved in stem cell self-renewal was reduced, indicating the importance of this pathway for both EMT and CSCs (Liu et al. 2012).

Another consequence of TGF-β signaling is the decreased expression of the $p15^{INK4b}$ and $p21^{CIP21}$ tumor suppressor genes, which act by stabilizing cyclin-dependent kinase inhibitors. It is thought that as the balance shifts between

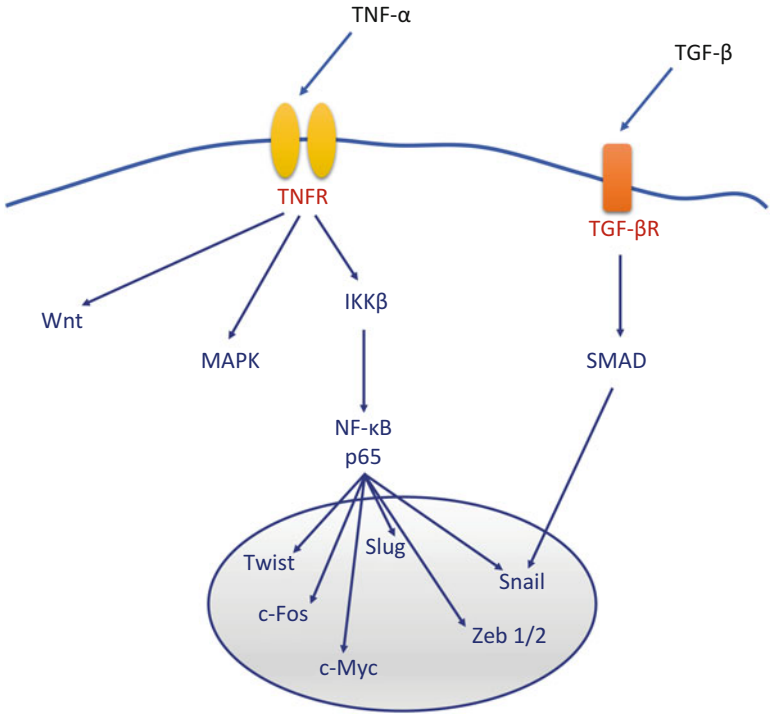

Fig. 3.3 *Signaling pathways influencing CSC phenotypes.* Simplified schematic of the signaling pathways downstream of TNF-α and TGF-β involved in key cancer stem cell phenotypes (Adapted from (Sethi et al. 2008; Balkwill 2009; Storci et al. 2010; Lee et al. 2012; Li et al. 2012a; Katsuno et al. 2013)

SMAD-dependent and SMAD-independent signaling, the cyclin-dependent kinase inhibitors fail to be induced, thus allowing cell proliferation to continue unchecked. However, at low levels of c-Myc, TGF-β typically induces the expression of p21, a cell cycle inhibitor, which in cancer cells, could contribute towards the CSC quiescent state (Kubiczkova et al. 2012). As well, Notch is activated in the immediate vicinity of active tumor progenitors, and Notch has been linked to p21 activation, thus suggesting that Notch, via p21, drives a quiescent phenotype consistent with CSCs, via some feedback control mechanism (Medema 2013). Once p21 is activated, several other signaling cascades are blocked, including c-Myc (Abbas and Dutta 2009) thus perpetuating the CSC phenotype. However, other pathways are activated which reduce these effects in some of the CSCs, thus leading to the acquisition of an EMT phenotype (Fig. 3.2).

While TGF-β can induce CSC phenotypes such as EMT, this effect has been shown to be transitory and required tumor necrosis factor (TNF)-α for more stable phenotypic changes (Mani et al. 2008; Asiedu et al. 2011). Moreover, TNF-α can up-regulate TGF-β expression at the transcriptional level and accelerate TGF-β induced EMT dramatically (Bates and Mercurio 2003).

4 Tumor Necrosis Factor-Alpha

Tumor necrosis factor (TNF) was first identified as a soluble factor released by host cells in response to a bacterial endotoxin that caused necrosis of tumors in both humans and animal models (Balkwill 2009). Amongst the large TNF superfamily, TNF-α has been recognized as a particularly important member (Balkwill 2009). TNF-α has a well-established pro-inflammatory function, shown to be an important mediator of the chronic inflammation of the bowel observed in irritable bowel disease (IBD). Indeed, TNF antagonists have been used quite successfully for the treatment of IBD, Chrohn's disease and ulcerative colitis (Balkwill 2009; Ben Musa et al. 2014), as well as rheumatoid arthritis, psoriasis, severe chronic asthma, ankylosing spondylitis and sarcoidosis (Balkwill 2009). Moreover, given its pro-inflammatory role, TNF-α has also been shown to act as a tumor initiator by stimulating the production of molecules that lead to DNA damage and mutations such as reactive oxygen and nitrogen species (RONS), and as a tumor promoter by altering cell proliferation and death (Hartnett and Egan 2012). TNF-α mediates its effects through binding to two different receptors with subsequent intracellular signaling occurring through several different pathways (Sethi et al. 2008; Egea et al. 2011). It contributes to malignant transformation through the up-regulation of *c-Fos* and *c-Myc* via NF-κB (Wang et al. 2013) (Fig. 3.3).

The role of TNF-α, however, is far from simple since it has also been shown to be cytotoxic and possess anti-tumor effects in several malignant diseases (Soria et al. 2011). The latter are thought to be related to its effects in destroying the tumor vasculature (Balkwill 2009), and may represent an effective therapeutic strategy early in cancer development (Ben Musa et al. 2014). However, it is now known that in advanced stages of cancer, the destruction of the tumor vasculature can lead to enhanced metastasis through EMT (Simon and Keith 2008; Mayol et al. 2009). Interestingly, it appears that low levels of TNF-α act as a tumor promoter and a recent study has shed light on how these low levels are maintained. The microRNA (miRNA) miR-130a, which has been demonstrated to promote cell survival in several cell lines, was shown to directly target the 3'UTR region of TNF-α, repressing its translation. Additionally, TNF-α stimulated enhanced miR-130a levels via NF-κB, providing a negative feedback loop that maintains TNF-α at levels that can promote tumor growth, at least in cervical cancer cells (Zhang et al. 2014). However, as shown quite eloquently in a previous study, ovarian cancer patients with the highest levels of TNF-α experienced the most intense down-regulation following monoclonal antibody therapy against TNF-α, although in mouse studies, a similar blockade of TNF-α resulted in decreased vasculature (Kulbe et al. 2012). Additionally, given that TNF-α therapy has been associated with an increased risk of malignancies, care must be taken when considering this as a therapeutic option (Bongartz et al. 2006).

This data suggests another potential link between TNF-α, EMT and CSCs. If blockade of TNF-α leads to a decreased vasculature, does this lead to a level of hypoxia required to promote CSC formation, and if so is this how miR-130a contributes to tumor promotion? In support of this, hypoxia is known to increase the number of $CD133^+$ CSC within a tumor mass, through the activation of Oct4 and

Notch signaling (Simon and Keith 2008; Mayol et al. 2009). Additionally, tumor hypoxia increases TGF-β secretion from cancer cells, thus triggering EMT (Jing et al. 2011). TNF-α can also cause the production of reactive oxygen species (ROS) from mitochondria under hypoxic conditions, and both ROS and NF-κB can facilitate EMT in certain cell types (Jing et al. 2011). As well, increased intracellular ROS levels may induce DNA damage within CSCs, resulting in additional mutations that promote disease progression (Tanno and Matsui 2011). Moreover, as described previously, the induction of Notch signaling leads to the activation of p21, resulting in quiescence (Medema 2013). Indeed, it is this link between TNF-α and Notch, whose activation occurs following prolonged exposure to TNF-α, that contributes to the CSC phenotype (Lee et al. 2012).

5 Interleukin-1

The Interleukin-1 (IL-1) cytokine family represent key mediators of inflammation and innate immune response, consisting of 11 cytokines and 10 receptors. Cells of the innate immune system, such as monocytes and macrophages are major sources of the two IL-1 cytokines, IL-1α and IL-1β, which both signal through the same receptor complex consisting of IL-1 receptor 1 (IL-1R1) and IL-1 receptor accessory protein (IL-1Rap). There are two mechanisms for controlling IL-1 signaling, either via the by competitive binding of the IL-1 Receptor antagonist (IL-1Ra) to the IL-1 receptor complex, or recruitment of a decoy receptor IL-1R2 that is unable to induce signal transduction (Dinarello 2011). Signaling pathways activated by IL-1 cytokines include myeloid differentiation primary response gene 88 (MYD88) and interleukin-1 receptor activated protein kinase (IRAK) as well as the NF-κB and PI3K pathways (Weber et al. 2010). The IL-1 family cytokines exist as an "inactive" uncleaved IL-1 which is cleaved to produce an "active" form that is able to signal via its cognate receptor complex (Werman et al. 2004).

Although cancer cells express IL-1α, its role varies depending on its state of cleavage. If IL-1α is uncleaved, it can act as a tumor suppressor, possibly via its ability to form a non-signaling complex with IL-1R1 that may act as an antigen for the immune system. Indeed, uncleaved IL-1α has been shown, at least initially, to reduce tumor load. Conversely, if IL-1α is cleaved it becomes a potent pro-inflammatory cytokine that favors tumor progression by promoting invasiveness and metastasis (Rider et al. 2013). Clearly, dual-function cytokines are unique as they do not require their cognate receptors to be expressed for autocrine signaling, and are able to elicit a function without their canonical signaling cascade. Therefore, dual function cytokines such as IL-1α require careful further study to delineate their exact roles in cancer.

Unlike IL-1α, IL-1β is not a dual function cytokine and requires cleavage of the "inactive" form before it becomes functional (Werman et al. 2004). IL-1β has been implicated in several types of cancer, including glioma, acute myeloid leukemia and colon cancer (Turzanski et al. 2004; Li et al. 2012b; Wang et al. 2012). The glioma

cell line, LN-229 lacks a CSC phenotype, but following addition of IL-1β and TGF-β increased its "stemness" changing it to a more CSC phenotype. This is of clinical relevance, since increased levels of both IL-1β and TGF-β occur in high grade gliomas with poor clinical outcomes for patients (Wang et al. 2012). Similarly, IL-1β can induces colon CSC self-renewal and increase invasiveness in cooperation with zinc finger E-box binding homeobox 1 (Zeb1) (Li et al. 2012b). IL-1β can also act as via antiapoptosis pathways to maintain the blast cells in acute myeloid leukemia (AML) (Turzanski et al. 2004). In addition, IL-1Rap is overexpressed in chronic myeloid leukemia stem cells with an IL-1Rap antibody able to induce antibody-dependent, cell-mediated cytotoxicity to these cells, but not those expressing low levels of IL-1Rap such as normal bone marrow cells (Askmyr et al. 2013).

6 Interleukin-6

The Interleukin-6 receptor (IL-6R) family consists of receptors composed of receptor chains related to the ligand-specific IL-6Rα or the archetypical GP130 (Boulay et al. 2003). The founding member, IL-6, is a pleiotropic cytokine that utilizes a receptor complex consisting of the ligand specific IL-6Rα and shared GP130 receptor subunits which signals via the downstream JAK2 and STAT3. One of the many roles of IL-6 is the maintenance of stem cells (Ernst et al. 1996; Notara et al. 2010). Therefore tight regulation of IL-6 expression and, in particular, its receptor IL-6Rα is required for normal development and homeostasis. The disruption of normal IL-6Rα expression is often pathogenic, and has been reported to have a role in several cancers, such as breast, ovarian and prostate cancers (Knupfer and Preiss 2007).

Serum IL-6 levels have been identified to correlate with poor prognosis for breast cancer patients (Sansone et al. 2007). One key role for IL-6 in breast cancer is to maintain the cancer stem cell population, with disruption of *IL-6* promoter methylation able to increase IL-6 levels and thereby increasing cancer stem cell maintenance (D'Anello et al. 2010). Furthermore, IL-6 is also able to induce EMT in breast cancer cells further enhancing their CSC properties (D'Anello et al. 2010). Breast cancers are a heterogeneous population of cells consisting of breast CSC and also differentiated breast cancer cells with the proportion of breast CSC and breast cancer cells varying depending on the microenvironmental conditions. The proportion of breast CSC to breast cancer cells is maintained by IL-6, a function that has also been observed in a prostate cancer cell line (Iliopoulos et al. 2011). Trastuzumab is a monoclonal antibody inhibitor for the v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2 (ERBB2) protein (also known as human EGF receptor 2, HER2), with overexpression of ERBB2 associated with aggressively proliferative breast cancer (Schroeder et al. 2014). Moreover, treatment of breast cancer with trastuzumab often leads to an enrichment of the breast CSC population with increased IL-6 expression. Antibody blockade of IL-6Rα is able to reduce the enrichment of the breast CSC population, leading to decreased tumor growth

(Korkaya et al. 2012). Therefore, combination treatment with a compound targeting non-stem cancer cells as well as a CSC targeting drug, such as Metformin, may be useful in avoiding CSC enrichment, with implications for the ability to successfully ablate the cancer (Hirsch et al. 2009; Iliopoulos et al. 2011).

7 Interleukin-8

Chemokine (C-X-C motif) ligand 8 or interleukin-8 (IL-8) is a proinflammatory chemokine and signals via two cell surface receptors, preferentially CXCR1 but also CXCR2 (Gales et al. 2013). These cell surface 7 transmembrane domain receptors are coupled with α, β and γ G proteins that induce intracellular signaling pathways that mediate cell survival, proliferation, angiogenesis and cell migration (Kobilka 2007), which include PI3K, RAS, JAK/STAT and WNT (Waugh and Wilson 2008). The CXCL8/CXCR1/CXCR2 signaling axis has been implicated in several cancers including melanoma (Singh et al. 2010), kidney (Liang-kuan et al. 2014), breast (Schillace et al. 2014) and pancreatic cancer (Chen et al. 2014).

CXCL8 is expressed by a wide variety of cells, particularly those of the immune system, and atypically by several cancers. Paracrine or autocrine signaling is achieved via CXCR1 (Chen et al. 2014) and CXCR2 (Liu et al. 2011) expressed in breast cancer cells, including CSCs. Indeed, increased CXCL8 serum levels have been shown to increase breast CSC activity, as judged by mammosphere formation (Singh et al. 2013). Similarly, CXCL8 and CXCR1 have been associated with pancreatic CSCs, with expression of CXCR1 correlating with that of pancreatic CSC markers, which has been linked with a lower survival rate due to metastasis of pancreatic cancer cells (Chen et al. 2014). The importance of CXCL8 signaling via CXCR1 has been demonstrated by inhibition of CXCR1, either by repertaxin, a small molecule inhibitor, or by a CXCR1 blocking antibody, which resulted in apoptosis of breast CSC and overall reduction of cancer load (Ginestier et al. 2010). Combined inhibition of CXCL8 and ERBB2, the latter of which is dysregulated in 25 % of breast cancers (Slamon et al. 1987), improves treatment in a synergistic manner (Singh et al. 2013).

8 The Central Role of NF-κB Pathway

The NF-κB pathway provides one of the major links between inflammation, CSCs and EMT. Several different stimuli can activate it. These factors include HIF-1α, which is induced in normoxic and cancer cells following stimulation by pro-inflammatory cytokines, such as TNF-α, IL-1β or IL-6 (Lu et al. 2006; Korkaya et al. 2012). Activation of the NF-κB cascade usually results in nuclear translocation and activation of p65 (Fig. 3.3). The p65 protein is a multifunctional transcription factor that elicits its physiologic function by regulating target gene expression upon

NF-κB activation. TNF-α, either from macrophages or the tumor microenvironment up-regulates the canonical NF-κB signaling through activation of IKKβ. Once up-regulated, activation of p65 follows and this protein then translocates to the nucleus where it induces Twist 1 expression thus promoting tumor metastasis via the EMT. This p65-induced EMT is accompanied by an increase in stem cell-like properties (Li et al. 2012a). Following the activation of the NF-κB pathway, several downstream events occur, such as the suppression of apoptosis. As well, the EMT regulators, Snail and Slug, are activated, thus promoting EMT (Korkaya et al. 2012) (Fig. 3.4).

It is interesting to note that NF-κB activation can stimulate different factors, depending on the cell of origin in the tumor. In basal-like breast cancer, NF-κB induces the expression of JAG1, leading to the Notch-dependent expansion of CSCs in a non-cell-autonomous manner (Yamamoto et al. 2013). However, it has also been shown that epigenetic mechanisms are regulated by NF-κB, with IKK-β regulating Lin28B, a RNA binding protein, which sustains the stemness of breast CSCs. This activation of Lin28B decreases Let-7 expression, leading to higher levels of IL-6, thus activating Stat3, and further stimulating NF-κB (Iliopoulos et al. 2011).

During inflammation NF-κB is able to promote the production of ROS, thus damaging DNA in surrounding epithelial cells, though in cancer this effect could cause the relevant mutations required for cancer cell continuation. Once NF-κB is activated

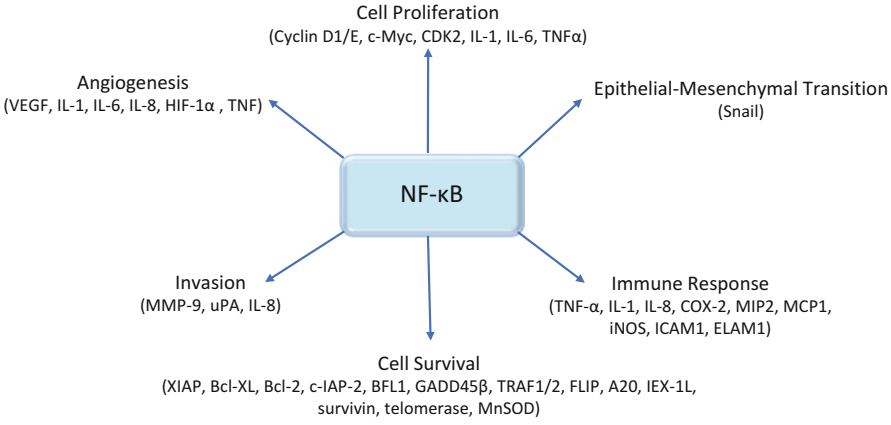

Fig. 3.4 *The diverse roles of NF-κB* (Adapted from (Baud and Karin 2009; Prasad et al. 2010; Li et al. 2012a)). *A20* zinc finger protein A20 (also known as TNFAIP3), *Bcl-2* B-cell lymphoma protein 2, *Bcl-XL* also known as Bcl-2 like 1, *BFL1* also known as Bcl2A1, *CDK2* cyclin-dependent kinase 2, *c-IAP-2* cellular inhibitor of apoptosis 2, *COX2* cyclooxygenase 2, *ELAM1* endothelial adhesion molecule 1, *FLIP* also known as casp8, *HIF-1α* hypoxia-inducible factor-1α, *ICAM1* intracellular adhesion molecule 1, *IEX-1 L* radiation-inducible immediate early gene (also known as IER3), *IL* interleukin, *iNOS* inducible nitric oxide synthase, *MCP2* monocyte chemoattractant protein 1 (also known as CCL2), *MIP2* macrophage inflammatory protein 2, *MMP9* matrix metalloproteinase 9, *MnSOD* manganese superoxide dismutase, *TNF* tumor necrosis factor, *TRAF1/2* TNF receptor-associated factor, *uPA* urokinase plasminogen activator, *VEGF* vascular endothelial growth factor, *XIAP* X-linked inhibitor of apoptosis protein

in gastric epithelial cells, it stimulates the transcription of IL-1, IL-6, IL-8, TNF-α and cyclooxygenase-2 (COX-2), which can further propagate the NF-κB activation response. How does this then lead to cancer progression, as this response to inflammation would be wound-healing, rather than promoting immortality? One suggestion is that some of its immune and inflammation-related target genes are not activated (Karin et al. 2002). It is also possible that mutations arise in the genes encoding the NF-κB/IκB family members that allows oncogenes to activate NF-κB (Karin et al. 2002; Karin 2009). One recent study investigated the regulation of the NF-κB pathway by miRNAs. This study found that in epithelial ovarian cells, Twist1 negatively regulated NF-κB dependent cytokine production (Yin et al. 2010). This seems counterintuitive as NF-κB increases Twist1 expression. However, this process is mediated by the microRNA, miR-199a which is frequently down-regulated during cancer progression (Fornari et al. 2010; Yin et al. 2010). Yin and colleagues went on to show, through knock-down experiments, that while Twist could influence IKKβ levels, TNF-α was required to induce RANTES production (Yin et al. 2010).

9 Therapeutic Prospects

While multiple factors are known to contribute to cancer formation and the cancer stem cell phenotype, cytokines are becoming recognized as one of the most viable targets for cancer therapeutics (Table 3.1). Given the positive feedback loops that drive CSC renewal, agents that can inhibit inflammatory cytokines or block inflammatory signaling pathways could potentially target and eradicate the CSC population. While there have been a number of disappointments, there have been a few successes.

There has been promising pre-clinical data from TGF-β-based therapeutics, the majority of these therapeutics caused harmful off-target effects which have prevented further clinical development (Perrot et al. 2013). TGF-β signaling begins when a ligand binds, and a type II receptor (TβRII) recruits and phosphorylates a type I receptor (TβRI). This TβRI is also known as an activin receptor-like kinase (ALK) and there are seven known type I ALK receptors, though ALK5 is the most specific for TGF-β (Mori et al. 2004). Targeting of the downstream ALK5 induced a range of toxicities, such as heart valve, hemorrhagic, degenerative and inflammatory lesions due to incomplete specificity. Galunisertib (LY2157299), developed by Eli Lilly, demonstrates cardiovascular toxicities (Gueorguieva et al. 2014), but has been used in several studies investigating whether it can sensitize CSCs to chemotherapy (Connolly et al. 2012; Perrot et al. 2013). It is also possible that this drug can be added to current therapeutic strategies to augment and enhance treatment regimens (Bhola et al. 2013) (Table 3.2).

Other options for targeting TGF-β include antisense oligonucleotides and monoclonal antibodies. Trabedersen (AP12009) is an antisense oligonucleotide that targets TGF-β2 mRNA (Schlingensiepen et al. 2011) which showed promise in phase I and phase II trials (Joseph et al. 2013). Efforts are being made to develop it

Table 3.1 Selection of therapeutic strategies for targeting cytokine networks

Name	Target	Type	Clinical trials	References
Galunisertib	Transforming growth factor receptor	Small molecule drug	III	Gueorguieva et al. (2014)
Trabedersen	Transforming growth factor-beta 2	Antisense oligonucleotide	III	Joseph et al. (2013)
Fresolimumab	Transforming growth factor-beta	Monoclonal antibody	II	Morris et al. (2014)
Infliximab	Tumor necrosis factor-alpha	Monoclonal antibody	III	Harrison et al. (2007), Brown et al. (2008)
Etanercept	Tumor necrosis factor-alpha	Neutralizing protein	III	Balkwill (2009)
N/A	Interleukin-1 receptor accessory protein	Monoclonal antibody	N/A	Askmyr et al. (2013)
Siltuximab	Interleukin-6	Monoclonal antibody	III	Jones et al. (2011)
Repertaxin	CXCR1	Small molecule drug	II	Ginestier et al. (2010)
Curcumin	NF-κB	Natural product	I	Gupta et al. (2013)
DTP3	NF-κB (GADD45β/MKK7)	Small molecule drug	Late 2015	Tornatore et al. (2014)

as a drug for systemic delivery rather than the prior intra-cranial infusion (NCT00761280). Fresolimumab (GC1008) is a human anti-TGF-β monoclonal antibody that has been investigated for the treatment of advanced malignant melanoma and renal cell carcinoma. In initial phase I trials, no dose-limiting toxicities were observed and preliminary evidence of anti-tumor activity was seen in 25 % of patients (Morris et al. 2014). Further studies are now being initiated for the treatment of metastatic breast cancer (NCT01401062).

TNF-α inhibition has fallen far short of expectations due to its high systemic toxicity (Burton and Libutti 2009). Clearly, care must be taken, as seen with the initial trials of TNF-α administration where systemic delivery was associated with severe toxicity and no, or limited, therapeutic effect (Mukaida et al. 2011). However, recent studies using an anti-TNF-α monoclonal antibody, infliximab, have shown disease stabilization in patients with advanced cancers (Harrison et al. 2007; Brown et al. 2008). Etanercept, a soluble TNFR2 fusion protein that binds and neutralizes TNF-α, was also found to stabilize disease in a minority of ovarian cancer patients (Balkwill 2009). There are currently ongoing clinical trials on both of these agents.

Due to the roles that interleukins play in other health and disease states, including inflammation, caution has also been exercised in the use of agents targeting IL-1, IL-6 and IL-8 in cancer. However, because of their role in other diseases, a wealth of information has been garnered from previous clinical trials. IL-1β has proven a successful therapeutic target in septic shock and rheumatoid arthritis, with the recombinant IL-1 receptor antagonist, Anakinra, having a remarkable safety record (Dinarello 2010). Clinical trials have been conducted with Anakinra in a

Table 3.2 Clinical trials involving LY2157299, an inhibitor of ALK5, lying downstream of TGF-β

Clinical trial identifier	Population cohort	Name	In combination with	Study completion date
NCT01722825 phase I	Japanese participants with cancer	A study of LY2157299 in Japanese participants with cancer		June 2014
NCT01682187 phase I	Malignant glioma	A dose-escalation study in participants with recurrent malignant glioma	Lomustine	December 2014
NCT01582269 phase II	Recurrent glioblastoma	A study in recurrent glioblastoma	Lomustine	December 2014
NCT01373164 phase Ib/II	Pancreatic cancer	A study in metastatic cancer and advanced or metastatic unresectable pancreatic cancer	Gemcitabine	March 2015
NCT02154646 phase Ib	Pancreatic cancer	A study of LY2157299 in participants with pancreatic cancer that is advanced or has spread to another part of the body	Gemcitabine	February 2015
NCT02240433 phase Ib	Hepatocellular cancer	A study of LY2157299 in participants with unresectable hepatocellular cancer	Sorafenib	February 2016
NCT01220271 phase Ib/IIa	Malignant glioma	A study combining LY2157299 with temozolomide-based radiochemotherapy in patients with newly diagnosed malignant glioma	Temozolomide	January 2016
NCT02008318 phase II/III	Myelodysplastic syndromes	A study of LY2157299 in participants with myelodysplastic syndromes		April 2016
NCT01246986 phase II	Hepatocellular carcinoma	A study of LY2157299 in participants with hepatocellular carcinoma	Sorafenib	October 2016
NCT02178358 phase II	Hepatocellular carcinoma	A study of LY2157299 in participants with advanced hepatocellular carcinoma	Sorafenib	December 2016

number of malignant disorders. One trial combined Anakinra with Dexamethasone to treat patients with smoldering or indolent multiple myeloma (NCT00635154), 1 patient achieved a complete response with Anakinra alone (n = 54), and when combined with desamethasone, 14 patients achieved a response to treatment (n = 29). Additionally, 49 patients out of the 54 were still alive and progression-free at 6 months, with a median duration of response recorded as 41.9 months, indicating some effectiveness. There are currently five other clinical trials currently recruiting to assess the efficacy of Anakinra in combination with other chemotherapeutic agents. However, as IL-1 plays a role in angiogenesis, care must be taken to develop appropriate clinical endpoints.

IL-6 targeting therapeutics have also been trialed in a number of malignant disorders, and have typically utilised monoclonal antibodies. CNTO 328 is a humanized monoclonal antibody (Guo et al. 2012). However, results have been limited. One study in patients with non-hormone responsive metastatic prostate cancer (NCT00433446) was not completed due to significant disease progression. Another clinical trial assessing the efficacy of CNTO 328 in myelodysplastic patients was terminated due to a lack of sufficient efficacy (NCT01513317). There is currently only one trial active, which will assess the efficacy of CNTO 328 in patients with high-risk smoldering multiple myeloma (NCT01484275). However, some efficacy has been observed in patients with renal cell carcinoma and ovarian cancer (Guo et al. 2012), suggesting it may be an effective therapeutic in some cancers, either as single or in combination therapy.

The use of IL-8 targeted therapies has been investigated in inflammatory diseases but to date, there is limited information on the efficacy in cancer. In vitro and in vivo studies have demonstrated inhibitory effects on tumor growth, angiogenesis and tumor dissemination, but these results have not be exploited in clinical trials (Skov et al. 2008). There is currently one pilot study investigating the safety profile in early breast cancer patients (NCT01861054) and an ongoing study to evaluate reparixin with weekly paclitaxel in patients with HER2-negative metastatic breast cancer (NCT02001974). Again, while safety has not proven to be an issue to date, only time will tell whether this therapeutic strategy is efficacious.

The NF-κB pathway is an intriguing transcription factor to target. This is because non-steroidal anti-inflammatory drugs, such as aspirin and salicylates, have been shown to be effective at inhibiting NF-κB activation (Karin et al. 2004). Indeed, cancer rates in aspirin users has been shown to be far reduced compared to the normal population (Cuzick et al. 2009). The most commonly accepted mechanism by which this is thought to occur is via inhibition of COX and thus prostaglandin production, although a prostaglandin inhibitor, indomethacin, failed to elicit an effect on NF-κB. Aspirin and salicylates do, however, inhibit some NF-κBs target genes (Karin et al. 2004). One of the issues with targeting the NF-κB is its role in inflammation, and thus any therapeutic should be transient so as not to cause immunosuppression (Baud and Karin 2009). Additionally, care must be taken so as to not enhance the production of IL-1β and related cytokines during bacterial infections, which has been a surprising side-effect of NF-κB inhibition (Greten et al. 2007; Baud and Karin 2009). While there are problems with targeting this pathway, there are a few drugs that have entered or are about to enter clinical trials.

Curcumin, derived from turmeric, is a natural product that has been shown to block IKK activation (Hussain et al. 2008). It has been well tolerated and there have been no associated toxicities. While there have been concern over the absorption of curcumin, bioavailability has been demonstrated in pancreatic cancer patients, although there remains no clinical trial on the effectiveness of this drug as a cancer therapeutic. Curcumin has been shown to decrease the levels of TNF-α, NF-κB, IL-6, IL-8, IL-10 and COX-2 in colorectal and pancreatic cancer, and multiple myeloma (Gupta et al. 2013) and may be beneficial given alongside other chemotherapeutics. Clinical trials are shortly to start investigating the potential of curcumin to prevent chemotherapy-induced fatigue in breast cancer patients about to receive radiotherapy (NCT01740323).

One approach that has been taken by scientists at Imperial College London was to investigate target genes downstream of NF-κB in an attempt to avoid some of the serious toxic side effects associated with other NF-κB targeted therapies. Tornatore and colleagues identified a protein complex, GADD45β/MKK7, that appeared to play a critical role in allowing cancer cells to survive. Using high-throughput screening, the investigators found two molecules that disrupted this protein complex with no toxicity to normal cells. This new drug, DTP3, will be entering clinical trials late in 2015 for the treatment of multiple myeloma (Tornatore et al. 2014).

10 Conclusion

Multiple factors in the tumor microenvironment contribute to the alteration of tumor cell function and behavior, including the transition to CSCs. CSCs represent a very fluid population of cells within the tumor mass that are reactive to environmental cues including cytokines secreted from both cancer cells and various tumor associated cell populations. Collectively, this microenvironment strongly influences progression of the tumor, with the influx of immune cells and their involvement in both paracrine and autocrine signaling directly contributing to metastatic disease. As a result, a new influx of therapeutics targeting these cytokine networks are being trialed, with some positive results being achieved. However, understanding of intricate details of gene regulation, such as via microRNAs and epigenetic changes, and how these impact remain largely unknown. More studies are required to further delineate these pathways to elucidate how cytokines that play a role in our immune response can be safely targeted to effectively eradicate cancer stem cells.

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Chapter 4
Implications of CXCR4/CXCL12 Interaction for Cancer Stem Cell Maintenance and Cancer Progression

Claudia Peitzsch, Monica Cojoc, Ina Kurth, and Anna Dubrovska

Abstract The chemokine receptor type 4 (CXCR4) is known to be involved in immunodeficiency disorders and contributes to different stages of cancer development. The CXCR4 expression level in cancer cells is an adverse prognostic indicator independent from other prognostic factors. Novel findings are pointing out the expression of CXCR4 in the tumor-initiating cancer stem cells (CSCs), which are involved in therapy resistance, relapse, metastasis and poor clinical outcome. CSCs are regulated by signals generated by the tumor microenvironment, but the exact mechanisms are not fully understood. Recent studies provide evidence for an important role of the CXCR4/CXCL12 axis for CSC maintenance, dissemination and metastatic colonization. In addition, this signaling pathway has a crucial contribution in modulation of the tumor microenvironment by inducing neo-angiogenesis and the recruitment of pro-tumorigenic myeloid cells to impede innate and adaptive immune mechanisms of tumor destruction. Moreover, binding of the chemokine ligand CXCL12 to its receptor CXCR4 has a direct effect on cell survival and growth of malignant cells. The correlation of CXCR4 expression with cancer stage and patient outcome makes CXCR4 an important prognostic marker as well as a druggable target with great potential for tumor sensitization to anti-cancer therapies.

Keywords Cancer stem cells • Resistance • Chemokine • CXCR4/CXCL12 axis

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1 Introduction

Chemokines are important paracrine and autocrine molecules involved in tumor development. CXCL12 and the signaling through its receptor CXCR4 affect tumor progression by controlling cell survival, proliferation and migration. Moreover, this CXCR4/CXCL12-depending signaling network is indirectly influencing the immune system and the neo-angiogenesis. The molecular and cellular heterogeneity of tumors hinders an improvement of cancer cure rate. In particular, the therapy-resistant subpopulation of tumor-initiating cancer stem cells (CSCs) is a cell subset within the bulk tumor responsible for dissemination, metastasis and relapse. This population shows an over-expression of CXCR4 in several cancers types, which is correlating with a high invasive potential and poor clinical outcome. This chapter is focusing on the recent findings about the role of the CXCR4/CXCL12 axis for tumor progression, CSC maintenance and the potential translation for therapeutic targeting.

1.1 Classification of the Chemokines and Their Receptors

Since 1970s, when the American physician and molecular biologist Robert J. Lefkowitz elucidated the structure and function of G protein-coupled receptors (GPCR), also known as seven-transmembrane receptors or heptahelical receptors, around 1000 types of GPCRs encoded by the human genome were identified. These receptors are located in the cell membrane, bind to extracellular substances like hormones, amines, neurotransmitters and lipids and transmit stimuli from these substances to an internal molecule called a G protein (guanine nucleotide-binding protein), therefore having a crucial role in signal transduction.

The chemokine family has as constituents small molecule (7–13 kDa) cytokines reported to mediate different pro- and anti-inflammatory responses and share a common biological activity as chemoattractants in stimulating the migration of different types of cells including lymphocytes, monocytes, neutrophils, endothelial cells, mesenchymal stem cells, and malignant epithelial cells (Smith et al. 2011; Viola and Luster 2008). Chemokines are typically ~70–80 amino acids in length and have at least four conserved cysteines, thus they are subdivided in four groups, according to the number and spacing of the N-terminal cysteine residues: CXC, CC, C and CX3C, where CXC chemokines have a single non-conserved amino acid residue (X) between the first N-terminal cysteine residues (C); CC chemokines have the two cysteine residues adjacent; C chemokines have only one N-terminal cysteine; while CX3C chemokines contain three non-conserved amino acid residues separating the N-terminal cysteine pair. Around 50 chemokines have been already described; most of them belonging to the CXC and CC classes, where several 6-cysteins CC chemokines were later introduced, which redefined the CC class. The CXC class could also be divided into glutamic acid-leucine-arginine (ELR) motif

containing ELR+, with Interleukin-8 or IL-8 (CXCL8) and ELR⁻, including Platelet Factor-4 or PF-4 (CXCL4), depending if the tri-peptide signature glu-leu-arg is N-terminal at the first cysteine.

Chemokines act by binding to the corresponding chemokine receptors (CCR), which currently include around 20 receptors, 10 of them being highly selective for one main high affinity (Kd ~ 1 nM) endogenous chemokine ligand (monogamous receptors) such as CXCR1, CXCR4, CXCR5, CXCR6, CCR6, CCR8, CCR9, CCR10, XCR1 and CX3CR1. The GPCRs encountered a recent classification system called GRAFS (Glutamate, Rhodopsin, Adhesion, Frizzled/Taste2, Secretin) (Bjarnadóttir et al. 2006), based on sequence homology and functional similarity: class A – rhodopsin-like, class B – secretin receptor family, class C – metabotropic glutamate/pheromone, class D – fungal mating pheromone receptors, class E – cyclic AMP receptors and class F – Frizzled/Smoothened.

In 2000, the chemokines and their receptors were introduced in a new nomenclature system, which changed their specific nomination to a harmonized classification and the chemokines ligands received a L and the receptors a R in their abbreviation, as IL-8 became CXCL8 or MIP-1α became CCL3, with the corresponding receptors CXCR or CCR. Thus, they are now designed from CXCR1 to CXCR5, CCR1 till CCR11, XCR1, and CX3CR1, based on their specific preference for certain chemokines.

1.2 Physiological Role of CXCR4/CXCL12

The C-X-C chemokine receptor type 4 (CXCR4), also known as fusin or cluster of differentiation 184 (CD184), belonging to Class A GPCR or rhodopsin-like GPCR family, is a seven transmembrane (TM) GPCR involved in multiple physiological processes in the hematological and immune systems, with a critical role in the development of different diseases like human immunodeficiency virus (HIV) infections, cancer, rheumatoid arthritis, pulmonary fibrosis, WHIM syndrome and lupus. CXCR4 is a 352 amino acid GPCR which was initially cloned as an orphan chemokine receptor and identified as one of the co-receptors for T-tropic HIV into $CD4^+$ T helper cells, and subsequently found to be expressed in a wide variety of tissues, including lymphatic tissues, thymus, brain, spleen, stomach and small intestine (Bleul et al. 1997, 1996; Oberlin et al. 1996). The sole universally accepted chemokine ligand for CXCR4 is CXCL12 (stroma-derived factor 1 alpha – SDF1α) a 8 kDa homeostatic chemokine peptide, mainly secreted by the bone marrow stroma cells. Although the CXCR4 has CXCL12 as unique ligand, CXCL12 itself can also bind to the orphan receptor CXCR7 (or atypical chemokine receptor 3, ACKR3), which is showing 10-fold higher affinity for CXCL12 than CXCR4 (Balabanian et al. 2005a). CXCL12 is sharing its binding sites to CXCR7 with CXCL11 (also known as interferon-inducible T-cell α chemoattractant, ITAC) that is also a ligand for CXCR3 (Singh et al. 2013).

CXCL12 was first described as an efficient lymphocyte chemoattractant and important regulator of hematopoiesis, significantly expressed in lung, colon, brain, heart, kidney and liver. Additionally, various tissues respond to different chemical or physical insults such as toxic agents, irradiation or hypoxia by increasing their expression of CXCL12, which is then able to recruit CXCR4-positive progenitor cells required for tissue regeneration (Kucia et al. 2004).

After CXCL12 binding, CXCR4 exerts its activity via a heterotrimeric G-protein, consisting of three subunits α, β and ɣ. Upon activation of basal form of the G-protein, the guanine nucleotide GDP is released and replaced by GTP, which leads to subunit dissociation into a βγ dimer and the α monomer to which the GTP is bound. The GTP is rapidly hydrolyzed to GDP resulting in reassociation of the receptor and the trimeric G-protein. $G\alpha_s$, one of the four subunits of the Gα complex ($G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12}$), stimulates adenyl cyclase, while $G\alpha_i$ inhibits adenyl cyclase. Further $G\alpha_q$ family acts via phospholipase C (PLC) to activate phosphatidylinositol-specific phospholipases, which hydrolyze PIP2 to generate two secondary messengers, IP3 and DAG which are capable of increasing the intracellular concentrations of free Ca^{2+}, and activate the transcription factor NF-κB through PYK2. Both $G\alpha_i$ and $G\alpha_q$ stimulate protein kinase B (AKT)/mitogen-activated protein kinases (MAPK) signaling pathway through phospholipase C (PLC)/protein kinase C (PKC)/Ca^{2+} or through $G\alpha_i$, which can trigger a signaling through extracellular signal-regulated kinases (ERK/1/2), leading to alteration of gene expression, actin polymerization, cell skeleton rearrangement and cell migration (Burger and Kipps 2006; Domanska et al. 2013). CXCR4-mediated chemotaxis is coordinated by phosphoinositide-3-kinase (PI3 kinase), which is activated by $G_{\beta\gamma}$ and G_{α} subunits, and associated with adhesion-dependent tyrosine kinase FAK and the anti-apoptotic AKT kinase, and is central for cell survival and proliferation (Fig. 4.1).

CXCR4 signaling is rapidly desensitized after ligand binding by receptor internalization upon which it is directed to the endosomal sorting pathway for ubiquitin-dependent degradation (Marchese and Benovic 2001; Marchese et al. 2003). The intracellular C-terminus of CXCR4 is swiftly phosphorylated at serine sites by G-protein receptor kinases (GRK) and this process is followed by recruitment of β-arrestin and clathrin-mediated endocytosis (Orsini et al. 1999) which then reduces CXCR4 coupling to $G\alpha_i$ signaling, favoring β-arrestin-mediated MAPK activation. The preferential signaling through G-proteins or β-arrestin is therefore influenced not only by the dimer formation of CXCR4 with CXCR7, but also by the oligomerization state of CXCL12 (Ray et al. 2012).

Being a homeostatic chemokine produced by bone marrow (BM) stroma cells, CXCL12 acts as major chemoattractant for the $CD34^+$ hematopoietic stem and progenitor cells (HSPC) and is involved in homing, retention and exit from other hematopoietic organs (Aiuti et al. 1997). Similarly, CXCR4 is widely expressed on $CD34^+$ HSPC, T lymphocytes, B lymphocytes, monocytes and macrophages, neutrophils and eosinophils, making the CXCL12/CXCR4 pathway responsible for the retention and homing of HSC in the bone marrow microenvironment and lymphocyte trafficking. Studies on CXCR4 deficient mice have shown failure in hematopoiesis, organ vascularization, and neuronal migration (Ma et al. 1998; Kazunobu

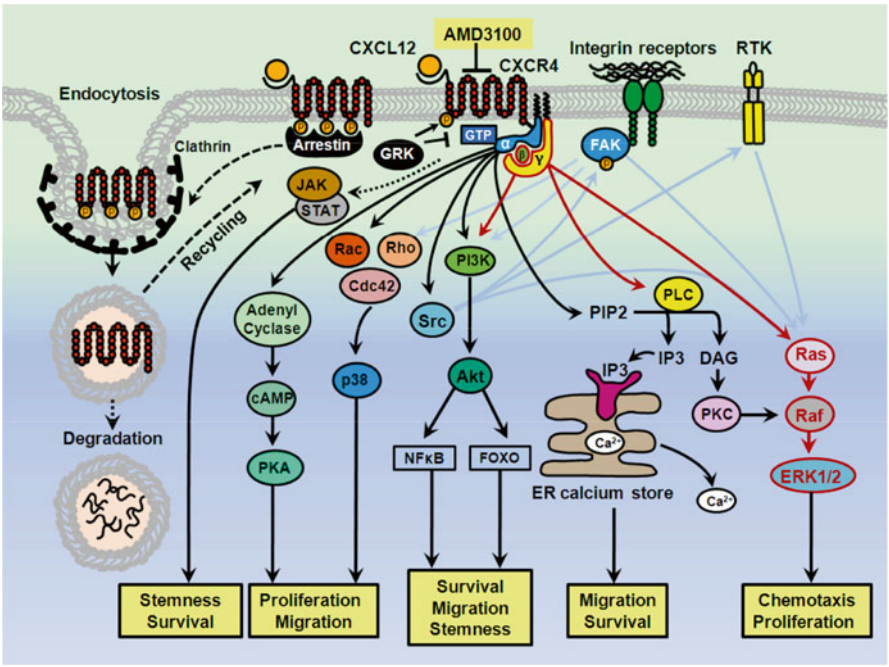

Fig. 4.1 A schemata of the CXCL12/CXCR4 signaling pathways (Reprinted with permission from Cojoc et al. 2013)

Tachibana et al. 1998a).CXCL12 knockout results in impaired hematopoiesis exhibited as a defect in trafficking of HSPC from the fetal liver to the embryonic bone marrow, as well as defects in heart and brain development, and vascularization. Thus, CXCR4 and CXCL12 knockouts are embryonic lethal (Ma et al. 1998; Ratajczak et al. 2006).

1.3 Pathophysiological Role of CXCR4/CXCL12

In the normal state, CXCR4 is expressed by cells of the nervous and immune systems, where it interacts with its ligand CXCL12, forming a vital complex required for formation of hematopoietic, nervous, vascular and cardiac systems during embryonic development (Nagasawa et al. 1996). Failure of CXCR4/CXCL12 interaction during incipient phase of embryonic development can lead to defects in bone marrow and cardiac function, with also repercussions in the adult life.

Therefore, in the WHIM syndrome (WS), a rare immunological disorder characterized by the presence of warts (W), hypogammaglobulinemia (H), bacterial infections (I) and myelokathexis (M) meaning an abnormal retention of pro-apoptotic

neutrophils in the bone marrow (Gorlin et al. 2000), the mutation of *CXCR4* gene causes truncation of the carboxyl-terminus (C-terminus) of the receptor, leading to a defect of CXCR4 inactivation (Hernandez et al. 2003). There were described patients carrying the heterozygous *CXCR4 1013* mutation, where CXCR4 failed to internalize on lymphocytes upon stimulation with the protein kinase C inducer phorbol-12-myristat-13-acetat (PMA), suggesting that the resistance to CXCL12-induced internalization could be caused by impaired agonist-dependent phosphorylation. In peripheral blood mononuclear cells (PBMC), refractoriness of CXCR4 for desensitization and internalization led to an enhanced CXCL12-promoted chemotaxis (Balabanian et al. 2005b). WS patients display high susceptibility to human papilloma virus (HPV) leading to skin lesions such as warts on hands, feet and trunk, genital and anal condylomas and mucosal lesions which often progress to carcinomas (Kawai and Malech 2009). Thus, the lack of CXCR4 inactivation is associated with an enhanced response to the chemokine, based on migration capacity criteria.

Idiopathic $CD4^+$ T-cell lymphocytopenia (ICL) is a rare hematological disorder characterized by a profound and persistent $CD4^+$ T-cell defect, which may mirror WHIM syndrome as a decrease of CXCR4 expression on $CD4^+$ T-cells, with impact on their differentiation and trafficking (Scott-Algara et al. 2010), exposing these patients often to life threatening opportunistic infections similar to those observed in acquired immunodeficiency syndrome (AIDS).

It was shown that CXCR4 is involved in B-cell production, myelopoiesis (Nagasawa et al. 1996), integrin activation (Peled et al. 2000), and chemotaxis (Bleul et al. 1996), and its up-regulation in multiple leukocyte subsets in systemic lupus erythematosus (SLE) patients is directly correlating the expression level with the aggressiveness of the disease (Guilpain et al. 2011). Moreover, the CXCR4/CXCL12 axis is critically involved in autoimmune response and end-organ inflammation in SLE (Chong and Mohan 2009).

CXCL12 is a potent chemoattractant for T-cells and a costimulator for their activation, with implications in rheumatoid arthritis (RA), where elevated CXCR4 expression by synovial memory T-cells is associated with accumulation of $CD4^+$ T-cells in synoviocytes, was reported (Buckley et al. 2000; Nanki et al. 2000). These data suggested that CXCL12/CXCR4 axis plays a role in the recruitment of inflammatory cells to the joint and both ligand and receptor have pro-inflammatory properties in human and mouse models of arthritis. The use of small molecule antagonists or CXCR4/CXCL12 knock-out mouse models induced a reduction of joint inflammation (Matthys et al. 2001). Increased production of CXCL12 by RA synovium leads to its accumulation and presentation on heparitinase-sensitive factors of endothelial cells, and participates in the angiogenesis associated with chronic inflammation (Pablos et al. 2003). In addition, CXCL12 was shown to increase the transcription of MMP-13, which may contribute to cartilage destruction during arthritis (Chiu et al. 2007). A pro-inflammatory activity of CXCR4 was also described in chronic lung inflammatory processes, when the CXCR4-positive cells influx from bone marrow to lung was observed (Gonzalo et al. 2000; Petty et al. 2007). However, in this case CXCR4 mediates its pro-inflammatory properties via neutrophil recruitment to the lungs, and not T-cells as for RA (Petty et al. 2007).

Since 1997, it was suggested that incorporation of bone-marrow-derived endothelial precursor cells (EPCs) into the new vessel lumen contributes to the angiogenesis and complements the resident endothelial cells (EC) in sprouting new vessels (Asahara et al. 1997). Along with development of this concept, it was discovered that the CXCR4/CXCL12 axis has angiogenic properties due to defective formation of blood vessels in gastrointestinal tract in mice lacking CXCR4 or CXCL12 (Kazunobu Tachibana et al. 1998a). CXCR4 activation stimulates the formation of capillary-like structures with human vascular endothelial cells, and CXCL12 up-regulates and synergizes with vascular endothelial growth factor (VEGF) which is able to increase the expression of CXCR4 and CXCL12, promoting a positive feedback loop for angiogenesis (Grunewald et al. 2006). The mechanisms of CXCR4 signaling which are known to up-regulate VEGF promoter activity (Pagès and Pouysségur 2005) include MAPK, ERK, JNK, and p38 kinase pathways (Busillo and Benovic 2007). The VEGF expression in ischemic tissues is directly regulated by hypoxia-inducible factor 1 (HIF1), and indirectly through CXCR4 signaling, which itself is increased by HIF1, VEGFA, and other factors such as PEA3, PAUF, PAX3-FKHR, nuclear respiratory factor 1 (NRF1), and estrogen (Schioppa et al. 2003; Sun et al. 2010; Zagzag et al. 2006). *In vivo*, CXCL12 induces angiogenesis in the rabbit cornea, in matrigel plugs in mice and in mouse models for wound healing and retinal ischemia (Deshane et al. 2007; Mirshahi et al. 2000). Moreover, CXCL12 stimulates the production of interleukin (IL)-8 in human umbilical vein endothelial cells (HUVEC), without affecting the production of other inflammatory mediators (Lin et al. 2000). IL-8 is a strong chemoattractant for neutrophils, which secrete various angiogenic molecules, including matrix metalloproteinases (MMPs). Therefore, the angiogenic response induced by CXCL12 may at least in part be mediated by IL-8 (Murdoch et al. 2008).

The involvement of CXCR4 and its ligand in injury-induced re-stenosis, which is a major problem after coronary re-vascularization, and in myocardial ischemia (MI) has been mostly attributed to resident cardiomyocytes and recruitment of circulating protective cells, like EPCs. CXCL12-induced cardioprotection was correlated with improved survival of hypoxic myocardium and increased neo-angiogenesis, through anti-apoptotic AKT and MAPK3/1 signaling in cardiac myocytes and endothelial cells (EC) (Hu et al. 2007; Saxena et al. 2008). Furthermore, CXCL12 triggered up-regulation of VEGF in the infarcted area and in cardiac EC (Saxena et al. 2008). The exogenous delivery of CXCL12 into the myocardium through local treatment with CXCL12-overexpressing adenovirus, CXCL12-transgenic skeletal myoblasts or CXCL12-releasing hydrogels was showing an enhanced recruitment of CXCR4 positive progenitor cells to the infarcted area in rodent models (Abbott et al. 2004; Purcell et al. 2012; Segers et al. 2007). Although CXCR4/CXCL12 axis plays a cardioprotective function, *CXCR4*-heterozygosity in mice reduced infarct size after MI, without affecting cardiac function, which can be explained by a counterbalance of reduced neo-vascularization and reduced inflammation with less neutrophils recruitment (Liehn et al. 2011). The same was observed for the adenovirus-mediated over-expression of CXCR4 in the heart. The infarct size was increased and cardiac function was reduced. Both effects has been associated with active recruitment of

inflammatory cells, enhanced tumor necrosis factor (TNF) α expression and increased apoptosis of cardiomyocytes (Chen et al. 2010). Taken together, the contradictory aspects of CXCR4/CXCL12 axis in inflammatory processes associated with ischemic heart leave room to more investigations for this affection.

In neurodegenerative diseases, a local vascular microenvironment is established in response to damaged tissue, and CXCL12 signaling influences local pathogenesis by regulating neural stem cells (NSC)-based tissue repair. This could also be demonstrated in the case of stroke within a murine model with temporary middle cerebral artery suture occlusion (MCAo). The migration of transplanted NSCs to lesion sites within this model is directly dependent on CXCL12/CXCR4 signaling (Imitola et al. 2004), probably mediated by DETA-NONOate, a nitric oxide donor, which directly up-regulates CXCR4 expression in peripheral stroma cells and coordinates their engraftment in the injured brains (Cui et al. 2007).

CXCR4 is also found to be highly expressed in several types of cancer like breast, ovarian, prostate and neuroblastoma (Müller et al. 2001a; Taichman et al. 2002; Teicher and Fricker 2010), therefore CXCR4/CXCL12 axis is more and more investigated as important regulator of tumor progression, angiogenesis, survival and metastasis and represents a crucial target in cancer treatment.

The bone marrow microenvironment, with the contribution of factors like CXCL12 or interleukin 6 (IL-6), enables the survival, differentiation and proliferation of normal hematopoietic cells, malignant hematopoietic cells and epithelial tumor cell bone metastasis, and sequesters tumor cells to this niche. Cancer cells take the advantage of the chemokine and their receptors expression to modulate the immune response to the tumor, when the simultaneous expression of CXCR4 and its ligand is acting as a pivotal autocrine/paracrine mechanism for attracting inflammatory, vascular and stroma cells to the tumor mass, where they are able to support the tumor growth by secreting growth factors, chemokines and pro-angiogenic factors. The CXCR4/CXCL12 implications in vascular development and stem cell homing upon tissue injury were already postulated (Ceradini and Gurtner 2005). Presently, a growing interest is focused on its role in angiogenesis, and vascular endothelial growth factor (VEGF) regulation. The first report of solid malignancies showed that CXCR4-positive breast cancer cells are responsive to CXCL12 (Müller et al. 2001a). In breast cancer cells CXCR4 expression is up-regulated by VEGF and making these cells responsive to CXCL12 (Bachelder et al. 2002). Inhibition of CXCR4 led to decreased angiogenic phenotype in vitro and in vivo.

Different in vitro studies described that growth factor CXCL12-dependent cell proliferation was correlated with phosphorylation of ERK1/2 and AKT in glioblastoma (Barbero et al. 2003), and with MIB1 proliferation index in the corresponding surgical specimen (Bajetto et al. 2007). Moreover, in the rat pituitary cell line GH4C1 CXCL12 was activating two intracellular pathways that independently contribute to cell proliferation, the Ca^{2+}-independent stimulation of the MAP kinase ERK1/2 activity (Lee et al. 2008). Moreover, in the rat pituitary cell line GH4C1 CXCL12 is activating two intracellular pathways that independently contribute to cell proliferation. One is the Ca^{2+}-independent stimulation via the MAP kinase ERK1/2 activity (Lee et al. 2008) and the second is the Ca^{2+}-dependent activation via the cytosolic

tyrosine kinase PYK2 and the large conductance Ca^{2+}- dependent K^+ channels (BKCa) (Florio et al. 2006). Consistently, the pharmacological inhibition of each of all these pathways indicated that all these intracellular transducers (Ca^{2+}, PYK2, BKCa and ERK1/2) are necessary for such an effect of CXCL12 (Florio et al. 2006). Chemokine involvement in tumor development was also shown in breast carcinoma, where CXCR4 over-expression was recognized as a requirement for breast cancer cell proliferation (Li et al. 2004) and silencing of CXCR4 causes a significant reduction of breast cancer cell proliferation in vivo and in vitro (Lapteva et al. 2004; Smith et al. 2004).

2 Deregulation of CXCR4/CXCL12 in Cancer

2.1 CXCR4/CXCL12 in Hematological Malignancies

As already described above chemokines have diverse cellular function like wound healing, cell recruitment, angiogenesis, cell trafficking, lymphoid organ development, inflammation, immune cell differentiation and metastasis. These fine-tuned processes are deregulated in cancer. Beside this physiological function of CXCR4 within normal tissue, CXCR4 is the most common chemokine receptor expressed on tumor cells and detected in over 20 different types of cancer (Balkwill 2004; Zlotnik 2006). The over-expression of CXCR4 and the enhanced intracellular signaling is linked to cancer aggressiveness and metastasis. Guo and colleagues showed the role of CXCL12 for cell survival and chemotaxis in murine embryonic stem cells and HSPC (Guo et al. 2005). Another function is the attraction of $CXCR4^+$ tumor cells to bone marrow niches via the CXCL12 gradient. High level of CXCL12 is expressed within hypoxic areas of tumors by carcinoma-associated fibroblasts (CAFs). The $CXCR4^+$ tumor cells compete with the normal HSPC for homing and retention within the bone marrow. These malignant cells are also able to displace HSPC from their proactive bone marrow microenvironment that results in hematopoietic dysfunction. The homing of leukemic cells to the BM niches provides them with their favorable growth and survival stimuli that protect them from chemotherapy-induced apoptotic signals (Zeng et al. 2006). This was found particularly for B-cell acute lymphoblastic leukemia (ALL) were the CXCR4/CXCL12 interaction, VLA4 expression and p38 MAPK activation are required for leukemia cell migration to CXCL12-secreting stromal cells in the bone marrow (Bendall et al. 2005; Burger et al. 1999; Sipkins et al. 2005). In acute myeloid leukemia (AML), CXCR4 expression level is depending on the differentiation stage. Undifferentiated (M0) and myeloid (M1/2) cells show a low CXCR4 expression, myelomonocytic (M4/5) and promyelocytic (M3) cells exhibit a high CXCR4 expression (Möhle et al. 2000). A CXCL12 gene polymorphism is associated with higher amounts of circulating AML cells and extramedullary disease (Burger and Bürkle 2007). In AML, CXCR4 is a prognostic marker of poor overall survival (Peled and Tavor 2013). A retrospective study assessed the CXCR4 expression in

90 BM samples from AML patients and demonstrated that the CXCR4/CXCL12 interaction is required for the survival of myeloid differentiating cells, while the co-expression of CXCR4 and CD34 is an indicator of a significant reduced patients' survival rate and a higher probability of disease relapse. Moreover, the CXCR4 expression was significantly higher in Flt3/ITD AML than in WT Flt3. Multivariate analysis of other previously established markers, such as age, LDH, leukocytosis and cytogenetic abnormalities indicated the predictive value of CXCR4 as independent prognostic marker of leukemia patients' survival (Rombouts et al. 2004). Similar results were found by Spoo and colleagues in a prospective AML study (Spoo et al. 2007). Patients with lower CXCR4 expression had a significant longer relapse-free survival (RFS) and overall survival (OS) than patients with intermediate or high expression level. Another retrospective clinical study by Konoplev et al. determined the prognostic impact of CXCR4 independent of Flt3 mutation in 122 AML patients (Konoplev et al. 2007). Moreover, the CXCR4 expression, the presence of multi-lineage dysplasia and high creatinine level are all together predictive for poorer overall and event-free survival.

In addition, CXCR4 is over-expressed in B-cell chronic lymphocytic leukemia (B-CLL) and contributes to the bone marrow tropism of B-CLL cells and heterotypic adherence to bone marrow stroma cells. Here it was found that stromal CXCL12 signals are an important regulatory factor for B-CLL cell survival. The adhesion of leukemic cells to CXCL12-expressing stromal cells is protecting them from apoptosis and increases cell survival, while CXCR4 antagonists re-sensitized CLL cells to fludarabine-induced apoptosis (Burger et al. 2000, 2005). In addition, the CXCR4 expression level is correlating with WBC counts, numbers of circulating CLL cells and disease stage (Burger and Bürkle 2007). Clinical trials found that a high CXCR4 level had a negative prognostic impact in 39 patients (Ishibe et al. 2002), but a large scale clinical study is expected to clarify the importance of CXCR4 as a predictor for disease stage and prognosis in leukemia patients.

During B-cell differentiation into plasma cells the chemokine responsiveness is changed and they become more sensitive to CXCL12. For example, multiple myeloma cells (MM) express functional CXCR4 that is cooperating with VLA-4 integrins for cell adhesion and migration. CXCR4 expression was also demonstrated in B-cell and T-cell non-Hodgkin lymphoma (NHL), which also expresses other chemokine receptors such as CXCR3, CXCR5, CCR7 and CCR5. Within this tumor subtype, CXCL12 is enhancing the migration of follicular NHL cells, while normal germinal center B-cells are not affected. Moreover it was found a distinct pattern of chemokine receptor expression involved in lymphoma cell trafficking and homing which allows distinguishing different NHL subtypes (Burger and Kipps 2006; Noll et al. 2012; Teicher and Fricker 2010).

The CXCR4 expression level can be used as a prognostic factor. For example in childhood ALL, CXCR4 level correlates with extramedullary organ infiltration and is thereby an independent predictor for peripheral lymphoblast count (Crazzolara et al. 2001). A retrospective study has shown that CXCR4 levels on ALL cells link to extramedullar organ infiltration and WBC count (Crazzolara et al. 2001; Schneider et al. 2002). A contradictory study by Kremer and colleagues showed that sole

CXCL12 is inducing a CXCR4-dependent apoptosis via Bcl-2 family members in AML cell lines and patient samples (Kremer et al. 2013). CXCR4 antagonists, such as Plerixafor (AMD3100) and T140 analogs, disrupt the adhesive tumor-stroma interaction and mobilize leukemic cells from their protective microenvironment making them more accessible for conventional anticancer drugs (Burger and Peled 2009). Several clinical trials showed that the disruption of the CXCR4/CXCL12 axis is a promising strategy to target leukemic cells, to overcome drug resistance and to improve the survival of leukemia patients.

2.2 CXCR4/CXCL12 in Solid Tumors

CXCR4 is also expressed in a broad range of normal and malignant non-hematopoietic tissues and is involved in the activation of different downstream signals depending on cell type and location. The over-expression of CXCR4 in malignant tissues compared to normal cells was particularly found in breast and lung cancer patients and is correlating with a poor prognosis (Fig. 4.2a, b). In addition, CXCR4 is the predominant chemokine receptor in ovarian cancer and is also implicated in other types of cancer such as prostate, colon and bladder. Within these cancers, CXCR4 expression was correlated with the metastatic capacity of cancer cells (Müller et al. 2001b). Further studies found a direct correlation of receptor up-regulation and tumor progression, neo-vascularization, invasion and metastasis in other tumor types (Kioi et al. 2010; Kozin et al. 2010; Ottaiano et al. 2006; Scala et al. 2005; Scotton et al. 2002; K. Tachibana et al. 1998b). For example, it is known that the CXCR4 expression is significantly associated with advanced differentiated renal cell carcinoma (RCC). Here it was found that in app. 70–90 % of all renal cell carcinoma (RCC) cases, CXCR4 and CXCL12 are over-expressed in tumor and vascular cells of RCC patients (Wehler et al. 2008). In addition, a positive correlation was demonstrated between a strong CXCR4 expression and poor survival in RCC patients treated with anti-VEGF therapies. In therapy-resistant tumors, the level of circulating cytokines are elevated. For example, a bevacizumab (Avastin) resistance in colorectal cancer (CRC) patients is characterized by up-regulation of CXCL12 and CXCR4 (Xu et al. 2009). Additionally, a high ubiquitous expression of CXCR4 has previously been demonstrated in small cell lung cancer (SCLC) cells in response to CXCL12 stimulation accompanied with an increased cell proliferation, adhesion and motility attributed to the activated PI3K signaling (Kijima et al. 2002). This high CXCR4 expression was also found in SCLC patients (Burger et al. 2003).

CXCL12 is constitutively expressed in lung, liver, skeletal muscle, brain, kidney, heart, skin and bone marrow and is induced by tissue damage, excessive bleeding, total body irradiation and chemotherapy. Moreover, it was shown that CXCL12 is implicated in recruitment of bone marrow-derived cells (BMDC) into tumors (D'Alterio et al. 2012; Hiratsuka et al. 2011; Tavor and Petit 2010). In vitro studies of breast cancer cells showed a high CXCL12 expression in stroma fibroblasts

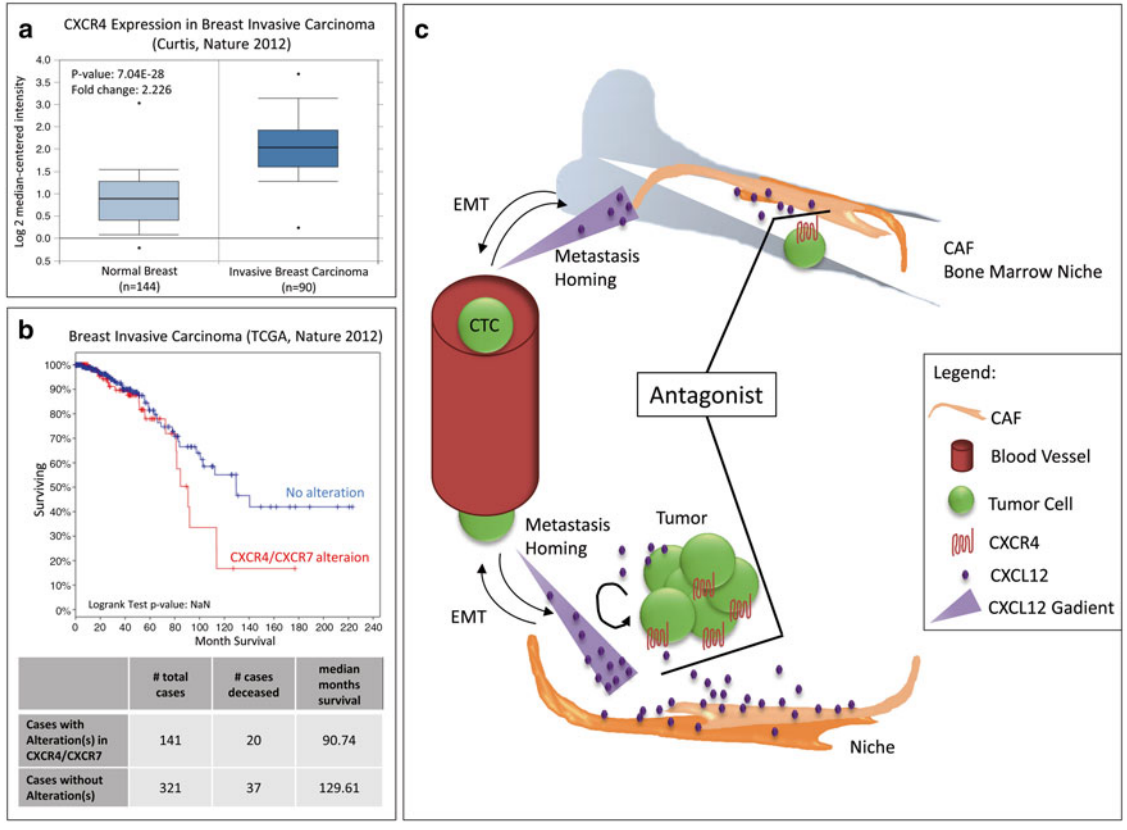

Fig. 4.2 (**a**) Oncomine database research reveals significantly up-regulated CXCR4 expression in invasive breast cancer (www.oncomine.org) (Curtis et al. 2012). (**b**) Over-expression of CXCR4 and CXCR7 in invasive breast cancer points to decreased survival rate of patients (n=463) as analyzed by the cancer genome atlas (TCGA, Nature 2012). The results shown here are in whole based upon data generated by the TCGA Research Network: http://cancergenome.nih.gov/. (**c**) Scheme of inhibition of CXCR4 mediated homing of cancer cells to metastatic niche sites

within metastasis of lymph nodes, lung, liver and bone marrow. In addition, CXCR4 is the major chemokine receptor in SCLC cells regulating their migration and invasion through the adhesion to bone marrow cells in a CXCR4- and integrin-dependent fashion. This adhesion protects SCLC cells from chemotherapy-induced apoptosis. Another study showed that CXCR4-expressing malignant cells interaction with CXCL12 induces retention or the metastatic spread of CXCR4-positive cancerous cells in CXCL12-rich tissues, such as bone marrow, lymph nodes, liver and lung as well as tumor growth (Müller et al. 2001b). In tumor xenograft models, CXCR4-expressing malignant cells stimulate neo-angiogenesis and metastatic dissemination (Balkwill 2004). Another study tried to answer the question about the role of CXCR4 for BM recovery after chemotherapy. Therefore, Sprague Dawley rats were treated with methotrexate (MTX) to disrupt the BM microenvironment. The myelosuppression and bone loss is affecting normal HSC proliferation, differentiation and maintenance. Moreover, these rats showed altered CXCL12 level and increased level of CXCL12-degradating MMP9 in the blood and BM (Georgiou et al. 2012). In addition to CXCL12 stimulation is the CXCR4 expression under normoxic conditions negatively regulated by the von Hippel-Lindau (VHL) tumor suppressor protein. This process is suppressed under hypoxic conditions within the tumor and is resulting in a HIF-dependent CXCR4 activation (Staller et al. 2003). Some growth factors such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) as well as transcription factors such as nuclear respiratory factor-1 are positive regulators of CXCR4 (Ishikawa et al. 2009; Phillips et al. 2005; Salcedo et al. 1999; Wegner et al. 1998; Zagzag et al. 2006). This complex regulation of CXCR4 expression is supposed to be an important process involved in cell transformation and tumor progression. Another process involved in neoplastic progression, tumor growth, angiogenesis and metastasis is the higher expression of CXCR4 in cancer-associated fibroblasts (CAFs) (Eck et al. 2009; Kojima et al. 2010; Orimo et al. 2005). These studies showed that soluble factors initiate the trans-differentiation of normal human mammary fibroblasts to tumor-promoting CAFs. These factors were identified as CXCR4 and MMP1 (Kwong et al. 2009). In addition, the CXCR4/CXCL12 interaction was identified to be critical for mesenchymal stem cell recruitment into breast and prostate cancer (Domanska et al. 2012; Olumi et al. 1999; Orimo et al. 2005). High intra-tumoral CXCL12 level attracts CXCR4-expressing inflammatory, vascular and stroma cells into the tumor that supports tumor growth by secretion of growth factors, cytokines, chemokines and pro-angiogenic factors.

A heavily discussed hypothesis about the process of tumor initiation, progression and metastasis is correlating the amount of so called cancer stem cells (CSCs) within a bulk tumor with the grade of malignancy and patient outcome. Recent studies focus on the identification of specific therapeutic and diagnostic CSCs markers. Nevertheless, the data on the functional properties of marker-positive cell populations remain controversial. Beside already accepted CSC marker like CD133 and aldehyde dehydrogenase (ALDH), CXCR4 expression has been detected in lung, pancreas and prostate CSCs (Bertolini et al. 2009; Hermann et al. 2007; Miki et al. 2007) and its over-expression relates to poor prognosis. In contrast, CXCR4 is

rarely expressed in normal tissue and the expression level is increasing during tumor progression due to its up-regulation by oncogenic signaling. Experimental stimulation of CXCR4 signaling by CXCL12 is increasing the CSC fraction within a bulk tumor cell population, while its inhibition is reducing the metastatic stem cell activity. Moreover, it was shown that CXCR4 activation has different effects on normal and malignant breast stem cells (Ablett et al. 2013). This makes CXCR4 a good targeting candidate for CSC-specific therapies (Cojoc et al. 2013) (Fig. 4.2c).

In summary, all these findings show that the CXCR4-CXCL12 interaction is crucial for chemotaxis and cancer cell movement to distant sites, and plays a role in cancer pathogenesis and metastasis.

3 Role of CXCR4 for Cancer Stem Cells

3.1 Impact of CXCR4 on Cancer Stem Cell Maintenance

Tumors are organized similar to normal tissue organs with differentiated malignant cells and stem cells, called cancer stem cells (CSCs), on the top of the differentiation tree. This was found by Dick and colleagues in the early nineties. Within their studies they could show that the $CD34^{+}CD38^{-}$ fraction of AML engrafted into immunocompromised mice was able to reconstitute the functional heterogeneity of AML (Bonnet and Dick 1997; Lapidot et al. 1994). This CSC hypothesis states that tumors consist of heterogeneous cell populations with different phenotype and functions forming the tumor bulk, while a small fraction of undifferentiated cells with self-renewal and multi-lineage differentiation potential are responsible for tumor recurrence, therapy resistance and metastasis (Baccelli and Trumpp 2012; Baumann et al. 2008; Medema 2013; Peitzsch et al. 2013). Novel hypothesis considers the existence of genetically and functional diverse CSC subclones within one tumor (Kreso and Dick 2014). This diversity can be also found within the $CD34^{+}CD38^{-}$ leukemic stem cell (LSCs) population (Kornblau et al. 2013; Sarry et al. 2011). The migration, homing and engraftment of LSCs in transplanted immunocompromised NOD/SCID mice are facilitated by their CXCR4 expression (Tavor et al. 2004). Moreover this up-regulated signaling in LSCs is leading to pro-survival signals, quiescence, and contributes to chemotherapy resistance (Riether et al. 2015). While the phenotypic definition of LSCs is well accepted, the search for proper CSC markers in solid cancer is still ongoing.

Beside established CSC markers such as CD133, ALDH and multi-drug resistance (MDR) proteins, CXCR4 is a promising candidate for isolation, targeting and monitoring of CSCs in patients during cancer therapy, choosing proper therapeutic strategies and improving patient outcome. Alongside with proliferation and survival, the CXCR4 axis plays a significant role in CSC self-renewal evaluated through sphere-formation, clonogenicity assays and limiting dilution assay in vivo. Moreover, CXCR4 expression is correlating with lymph node metastasis and poor prognosis of various cancer types (Kato et al. 2003; Kim et al. 2005; Schimanski

et al. 2006). In particular in NSCLC, CXCR4/CXCL12 seems to be critical for metastasis (Belperio et al. 2004; Jung et al. 2012; Su et al. 2005). For several cancer types, including glioma, prostate cancer, pancreatic adenocarcinoma and breast cancer, the cell surface expression of CXCR4 is a putative marker for CSCs (Ehtesham et al. 2009; Hermann et al. 2007; Miki et al. 2007). Studies from our own group showed that the CXCR4 is highly up-regulated in prostate $CD44^+CD133^+$ CSCs and is crucial for CSCs self-renewal, differentiation, cell adhesion and tumorigenicity (Dubrovska et al. 2012a). Moreover, we could show that chemoresistant breast cancer harbor a high percentage of CSCs, which are maintained by a cross-talk of the CXCR4 and aryl hydrocarbon receptor (AhR) signaling (Dubrovska et al. 2012b). This is in agreement with other studies were enhanced CXCR4 signaling is able to drive ER^+ breast cancer to a metastatic and therapy-resistant phenotype (Rhodes et al. 2011). A study of glioblastoma is indicating that CXCR4 is mediating the proliferation of glioma CSCs, but not other malignant cells within the bulk tumor (Ehtesham et al. 2009).

CSCs share functional and phenotypic characteristics with normal stem cells. For example stemness and high tumorgenicity is promoted by several factors including IGF1R, VEGF, TGF-β signaling secreted by CSCs themselves, other malignant cells within the bulk tumor or immune cells and CAFs within the surrounding tissue (Chen et al. 2014; Hasegawa et al. 2014; Seton-Rogers 2012). In addition, several studies could show that the up-regulation of CXCR4 on CSCs for example in non-small cell lung cancer (NSCLC) is important for the maintenance of stemness and drug-resistance (Jung et al. 2012), while the inhibition of the CXCR4-CXCL12 signaling by AMD3100 is reducing the self-renewal and survival of GBM CSCs (Gatti et al. 2013). In contrast, another study found that $CD133^+CXCR4^+$ colon CSCs correlate with high metastatic potential and poor prognosis, but that the CXCR4 expression on CD133-positive cells is not influencing the stemness properties of the colon cancer cell line HCT116 (Zhang et al. 2012). CSCs can be also isolated as so-called side population (SP), which includes cells with a high efflux capacity. Within this population Van den Broek and colleagues found that the gene expression level of CXCR4 and ABCB1 in primary pancreatic ductal adenocarcinoma (PDAC) is correlating with worse patient outcome and demonstrated that these CSC-associated genes have a high prognostic value (Van den Broeck et al. 2013).

Another feature of CSC is the genomic, phenotypic and functional plasticity leading to the cellular heterogeneity within the cancer (Pardal et al. 2003). One example of such plastisity is the trans-differentiation of epithelial to a mesenchymal phenotype, called EMT, which is required under physiological conditions for tissue morphogenesis during embryonic development. This process is regulated by several cytokines and growth factors, such as TGF-β, and causes the gain of invasive and metastatic properties (Thiery et al. 2009). A study by Akunuru et al. found that NSCLC $SP^+CD133^+ALDH^+$ CSCs of patient-derived primary tumor cells undergone EMT, which was activated by high Rac1 GTPase activity. These cells showed high activity of metastasis-associated genes, including CXCR4, TNF-α, VEGFA and HoxB9 (Akunuru et al. 2012). Within heterogeneous tumor bulk populations a clonal selection of populations primed for metastatic spreading occurs. This was

shown for example by a study form Zhang et al. using primary triple-negative breast cancer. They found that the CAF-derived factors CXCL12 and IGF1 within the tumor stroma select for bone metastatic cells with high Src and PI3K/AKT activity and determine the bone-specific metastatic tropism (Zhang et al. 2013b). They hypothesized that stroma signals resemble those from the distant organ and cells were primed for metastasis to this organs. Moreover, by comparing malignant cells from the primary tumor and their distant metastasis they could illuminate the evolution of cancerous cells through the metastatic traits (Nguyen et al. 2012; Valastyan and Weinberg 2011). High-resolution sequencing and other approaches provide evidence that this metastatic process relies on epigenetic amplification of cell survival and self-renewal mechanisms rather than on driver mutations (Oskarsson et al. 2014; Vanharanta and Massagué 2013). But for this process CSCs need to find supportive sites, where disseminated cancer cells are able to home.

3.2 Influence of CXCR4/CXCL12 Signaling in the Cancer Stem Cell Niche

In line with the CSC niche theory, where tumors are hypothesized as abnormal organs with patterns of normal organs, it was observed that CSCs reside within specified CSC niches, which control their self-renewal and differentiation (Borovski et al. 2011). Within these niches CSCs come into close contact with supportive cells and other microenvironmental factors, which are providing information concerning cell trafficking, tumor expansion, recurrence and metastasis. Within this network CXCL12 is a multifunctional cytokine and is secreted for example by niche endothelium and stroma cells (Dar et al. 2005). Novel hypothesis of cancer progression and metastasis propose that the transforming events in solid tumors occur in hypoxic cancer cells within specific niches. This is followed by an up-regulation of HIFs and CXCR4 and an induction of stem cell-like phenotypes, which in turn induces the EMT program within hypoxic regions at the invasive front of primary prostate and breast cancer and may lead to their invasion and dissemination. Beside the hypoxic niche with more quiescent CSCs, a perivascular niche around blood capillaries exists. This niche was identified especially for glioma stem cells and is supplied by the Hedgehog, Notch and PI3K signaling (Charles and Holland 2010; Hambardzumyan et al. 2008). Also breast and lung cancer cells, which infiltrate the brain place themselves preferentially around capillaries (Carbonell et al. 2009, p. 200; Kienast et al. 2010). These stem cell niches are the source for developmental and self-renewal signaling, such as Wnt, Notch, TGF-β, Hedgehog and CXCL12 pathways, which overlap with those from adult stem cell niches (Hsu and Fuchs 2012; Merlos-Suárez et al. 2011; Moore and Lemischka 2006; Morrison and Spradling 2008; Takebe et al. 2011).

One source of these signals is the CXCL12 produced by mesenchymal cells in the bone marrow, where it provides chemotaxis, PI3K-mediated survival signals and a niche for CXCR4-overexpressing bone metastatic CSCs (Müller et al. 2001b;

Zlotnik et al. 2011, p. 201). Under physiological condition, such as the HSC homing, the CXCL12 gradient restricts the homing of HSCs within the bone marrow (Wright et al. 2002). This process is regulated by the tissue hypoxia within the marrow microenvironment and induces the production of hypoxia-inducible factor-1 (HIF-1), resulting in selective expression of CXCL12/CXCR4 and increased cell adhesion, migration and homing of circulating $CXCR4^+$ progenitor cells (Ceradini et al. 2004). The HSC maintenance is provided by the hypoxic HSC niche in the BM along stromal cells. These stromal niches are scattered throughout the intertrabecular space adjacent to the vascular network, the sinusoids. Stromal cells within the niche provide attachments sites via very late antigen 4 and 5 (VLA4/5) and growth factors for HSPC growth and differentiation. But so far the molecular mechanisms for stem and stroma cell interaction are not fully understood.

One study in HNSCC patient samples found CXCR4 and CXCL12 expression in $CD44^+$ CSC tumor nest, where CD44-expressing cells were located at borderline of tumor nests, but not in the tumor stroma. In this tumor entity the CXCR4-CXCL12 interaction is a crucial pathway of CSC trafficking into CSC niches. Moreover, the authors found a higher CXCL12 concentration in HNSCC patients compared to healthy humans. This is making CXCL12 for HNSCC patients a promising tumor marker (Faber 2013). Also in glioblastoma (GBM) $CD133^+nestin^+$ CSCs an overexpression of CXCR4 was detected (Singh et al. 2004). Moreover, within this tumor entity it was found that the chemokine CXCL12 was secreted by the CSCs themselves for autocrine stimulation (Gatti et al. 2013; Salmaggi et al. 2006). This CXCR4 activation in combination with VEGF and HGF signaling under hypoxic conditions is the key factor driving the tropism of normal stem cells towards gliomas (Zhao et al. 2008). But a recent study by Liu et al. revealed a high heterogeneity of CXCR4 expression within different GBM CSC cultures (Liu et al. 2013; Wurth et al. 2014).

3.3 Role of CXCR4 for Circulating Tumor Cells

As part of the metastatic cascade malignant cells lose the cell-to-cell contact and undergo EMT for extravasation into the blood stream. These epithelial cell adhesion molecule (EpCAM)-positive circulating tumor cells (CTCs) and/or circulating CSCs have a prognostic value for tumor development and metastasis (Braun et al. 2005; Cristofanilli et al. 2004). CTCs detected in the peripheral blood can originate either from the primary tumor or from metastasis. The quantification of CTCs during cancer therapy allows to monitor the responsiveness to the treatment using a non-invasive method. Recent studies postulate that CTCs exhibit a stem cell-like phenotype and express several stem cell and EMT markers, such as ALDH1, CD44, CD133, fibronectin or N-cadherin (Krawczyk et al. 2014).

Circulating prostate and breast cancer cells express high levels of CXCR4 and can preferentially disseminate and home to specific metastatic sites, such as bone through the chemoattractive CXCL12 gradient formed by endothelial cells.

The hypoxia-adapted CSCs may compete with LT-HSC for the hypoxic endosteal niche within the BM and survive under a dormant state for a long period of time. The activation of dormant CSCs may occur through the release of different growth factors and cytokines by cancer cells and stromal cells within the tumor microenvironment (Mimeault and Batra 2013). Gradients of CXCL12 attract CTCs to secondary organs, support proliferation of malignant cells and contribute to characteristic metastatic pattern (Müller et al. 2001b). These activated CSCs can give rise to a total tumor cell mass and skeletal metastasis. A recent study found CXCR4 expression also in blood samples of 82 % melanoma patients (40/49) with 1 CTC per 10 ml blood, but no correlation of chemokine receptor expression and clinical response or metastatic pattern was observed (Fusi et al. 2011). This group showed already in a previous study that $EpCAM^+$ and intracellular cytokeratin-postitive CTCs isolated from patients with solid tumors expressed the chemokine receptors CXCR4, CCR6, CCR7 and CCR9, but they couldn't find a correlation with the metastatic pattern and the number of circulating CTCs in the peripheral blood (Fusi et al. 2012). A molecular regulation of early extravasation of metastatic tumors cells in vivo was functional characterized by Gassmann et al. using human liver metastatic HEP-G2 hepatoma and HT-29LMM colon cancer cells. They dissected the metastatic cascade using intravital fluorescence microscopy and studied the interaction of tumor cells with the microsystem of the liver as major metastatic organ. Here they found that the rate-limiting event for metastasis is the tumor cell extravasation by chemokine ligand – receptor interaction (Gassmann et al. 2009). Within another study by Markiewicz et al. authors isolated CTCs from blood samples of breast cancer patients with and without lymph node metastasis. They found that tumors of patients with lymph node metastasis have CTCs with superior seeding and metastatic potential in comparison to node-negative patients. This is correlating with an enhanced VIM, uPAR and CXCR4 expression and a mesenchymal phenotype (Markiewicz et al. 2014). It was also reported that the cytoplasmic CXCR4 expression is associated with advanced colorectal cancer (CRC) and breast cancer, lymphovascular invasion, lymph node metastasis and poor prognosis (Chen et al. 2013; Wang et al. 2010, p. 4; Yasuoka et al. 2008). The metastatic process is widely expected to be mediated through CTCs expressing adhesion molecules that actively bind to vascular endothelial cells, e.g. to E-selectin, for intra- and extravasation (Burdick et al. 2012). Although CTCs are used in several clinical trials, many issues regarding their detection and characterization remain unresolved (Alix-Panabières and Pantel 2014). Moreover, studies linking CSCs to CTCs and metastasis are missing so far.

3.4 Impact of CXCR4/CXCL12 Signaling for Metastasis

The metastatic cascade involves interplay between altered cell adhesion (e.g. CAMs, cadherins, integrins), survival (e.g. IGF), proteolysis and ECM remodeling (e.g. MMPs, uPA, ADAMs, heparanase), migration (e.g. Met-SF/HGF, FAK), lymphangiogenesis,

angiogenesis (e.g. VEGF, PDGF, bFGF), immune escape (e.g. MHC loss) and homing to the target organ (e.g. chemokine receptors, CD44, osteopontin) (Bogenrieder and Herlyn 2003). This metastatic process involves as key event chemotaxis, which is the coordinated trafficking and organization of cells and is regulated by the interaction between the chemokines and their corresponding receptors. For examples, chemokines on the luminal surface of the vascular endothelium are able to activate chemokine receptors on blood lymphocytes leading to the rolling process and extravasation. In particular, this interaction is leading to polymerization and breakdown of actin and the formation of lamellipodia (Baggiolini 1998).

Chemotaxis can be also stimulated by the activation of integrins. They cause leukocyte adhesion to endothelial cells, trans-endothelial migration and homing towards chemokine gradients. One important chemokine-receptor interaction for this process is the CXCR4/CXCL12 axis. Several clinical studies showed that the CXCR4 expression on cancer cells is correlating with poor clinical outcome. For example, breast cancer cells metastasize preferentially to CXCL12-secreting organs, such as lung, bone and lymph nodes (Kang et al. 2005; Mukherjee and Zhao 2013). There are studies showing an involvement of CXCR4 in metastasis of colorectal cancer and extra-nodal recurrence (Kim et al. 2005; Kucia et al. 2005; Schimanski et al. 2006, 2005). As already mentioned above the metastatic activity of pancreatic cancer cells is based on the $CD133^{+}CXCR4^{+}$ CSCs (Hermann et al. 2007). This migratory and invasive potential of pancreatic CSCs could be experimentally decreased by down-regulation of CXCR4 in a co-culture system with pancreatic stroma cells (Moriyama et al. 2010). Also in other tumor entities, such as GBM, it was shown that the chemokine receptors CXCR4 and CXCR7 are over-expressed in GSCs and this correlates with their invasive potential. The activation of the orphan receptor CXCR7 is scavenging CXCL12 and is leading to the lysosomal degradation of CXCL12. This promotes metastasis of CXCR4 positive breast cancer cells in an orthotopic tumor model and showed that the therapeutic CXCR7 inhibition and chemokine scavenging is limited by the $CXCR4^{+}$ CSC growth (Luker et al. 2012). In contrast, in breast cancer it was shown that CXCR4 and CXCR7 having opposing roles on metastasis. CXCR7 alone had no effect on chemotaxis and invasion, while in combination with increased CXCR4 expression, the matrix degradation via MMP12 and the chemotaxis was enhanced (Hernandez et al. 2011).

The molecular mechanisms how CXCR4 expression is up-regulated in cancer and how this elevated expression is regulating the metastatic properties of malignant cells is not well understood. One study showed a direct transcriptional regulation of CXCR4 by c/EBPβ and liver-enriched inhibitory protein (LIP) (Park et al. 2013). Another study found that CXCR4 induced the metastatic phenotype of breast cancer cells via CXCR2, MEK and PI3K signaling. This was shown using a constitutive active CXCR4 variant in MCF7 breast cancer cells. This cell line showed enhanced EMT phenotype with enhanced expression of CXCR2, CXCR7, CXCL1, CXCL8, CCL2, IL-6 and GM-CSF, ZEB-1 up-regulation, E-cadherin loss, up-regulation of catenin and ERK1/2 and MMP2 activation (Sobolik et al. 2014). The understanding of the molecular basis of CXCR4-mediated cancer cell metastasis is a potential novel strategy to reduce incidence and mortality of cancer patients.

4 Involvement of CXCR4/CXCL12 in Therapy Resistance

All the above mentioned mechanisms of cancer, such as self-renewing CSCs, malignant CTCs in the blood stream, specified protective niches and metastasis to target organs, are gained by the malignant cells during tumor progression and provide the tumor bulk population several possibilities to circumvent chemo- and radiotherapeutic treatment. Additional mechanisms are drug exclusion, drug metabolism and alteration of the drug target. This acquired drug resistance depends on several genetic and epigenetic alterations and provides malignant cells a possibility for dynamical functional and phenotypic switching making specific cancer cell populations hard to target and to eradicate. This are challenging novel improvements for the development of anti-cancer drugs (Michor et al. 2006).

Beside the involvement in CSC regulation the CXCR4/CXCL12 interaction is critical for therapy resistance by direct stimulation of cancer cell survival and invasion, recruitment of myeloid bone marrow-derived cells to facilitate tumor recurrence and metastasis and by promoting angiogenesis (Duda et al. 2011; Teicher and Fricker 2010). Similar mechanisms were found by Singh and colleagues in pancreatic cancer cells. Within these cells CXCL12 induced the activation of FAK, ERK, AKT, β-catenin and NF-κB signaling and conferred resistance to gemcitabine (Singh et al. 2010b). Several reports have proven that over-expression of CXCR4 significantly correlates with chemotherapy resistance. Also in ovarian cancer, the CXCR4 expression is a prognostic factor for cisplatin-based chemotherapy response, where high CXCR4 expression is associated with cisplatin resistance, poor progression-free survival and low overall survival (Li et al. 2014). In NSCLC, HNSCC and prostate cancer patients genetic alterations of the *CXCR4* and *CXCL12* gene leading to an increased CXCR4/CXCL12 signaling within these patients (Hirata et al. 2007; Lee et al. 2011; Teng et al. 2009; Wald et al. 2013).

Another problem is the chemotherapy-induced up-regulation of CXCR4. This was found for example in pediatric AML by enhanced CXCL12 secretion, chemotaxis and stromal protection from apoptosis (Sison et al. 2013). Other inducers of CXCR4 are tumor hypoxia via HIF-1α and STAT3 signaling and this hypoxia-induced CXCR4 is conferring resistance to anti-angiogenic therapy via targeting VEGF (Locasale and Zeskind 2012). The mechanisms behind this resistance are considered to be the attraction of $CXCR4^+$ myeloid cells, which are responsible for mediating VEGF-independent angiogenesis (Du et al. 2008).

Beside chemotherapy radiotherapy is a key treatment modality for cancer patients. App. 50 % of all cancer patients are treated with radiotherapy to reduce tumor burden, but also here is the resistance to radiotherapy a major problem. Recent evidence suggested that the CXCR4/CXCL12 axis is also involved in radioresistance by inducing microenvironmental changes around the bulk tumor, recruition of CD11b-positive immune cells, up-regulation of the PI3K/AKT signaling, stimulation of neo-vascularization and induction of migration. Our own data show that radioresistant prostate cancer cells are maintained by CXCR4 signaling. Moreover, prospectively isolated $CXCR4^+$ CSCs are less radiosensitive than

CXCR4-depleted DU145 prostate cancer cells in a standard radiobiological colony-formation assay. The $CXCR4^+$ cells co-localize with $ALHD^+$ CSCs and hypoxic HIF-1 expressing cells within a subcutaneous xenograft model of DU145 cells in NMRI nu/nu mice (Peitzsch et al. 2014; Trautmann et al. 2014). The radioresistance of CXCR4-positive cells in prostate cancer was proven by Domanska et al.. This study showed that in a pre-clinical prostate cancer model using immunocompromised mice CXCR4 inhibition by AMD3100 is sensitizing the xenograft to irradiation. But an unexpected finding was the additional induction of cancer cell mobilization from the subcutaneous bulk tumor and metastatic lesions in the kidney, axillary lymph node and subcutaneous nodules (Domanska et al. 2014).

All above mentioned pre-clinical and clinical studies confirmed that CXCR4 and CXCL12 are promising therapeutic targets to sensitize cancer cells to chemo- and/or radiotherapy.

5 Therapeutic Targeting of CXCR4/CXCL12 Pathway

The strongly outlined role of CXCR4/CXCL12 signaling as one of the key stimuli involved in the interaction between tumor cells and their microenvironment has stimulated an intensive study of the modulators of this pathway, which block interaction between CXCR4 receptor and its ligand CXCL12. Multiple agents targeting CXCR4/CXCL12 in tumor cells were developed during the last decade. All these modalities can be roughly divided to four groups: small molecule antagonists of CXCR4, peptide antagonists of CXCR4, antibody against CXCR4 and glycosaminoglycans (GAGs) mimetics (Weitzenfeld and Ben-Baruch 2014).

Nevertheless, to date only few compounds targeting CXCR4/CXCL12 pathway have been advanced to the early stages of clinical trials and only one chemical drug AMD3100 (also known as Plerixafor and Mozobil) was approved by FDA for stem cell mobilization in patients with lymphoma and multiple myeloma (Pusic and DiPersio 2010). AMD3100 was first discovered as anti-HIV agent and then it was found to be a potent inducer of "mobilization" of hematopoietic stem cells from the bone marrow to the bloodstream that is currently used in leukemia patients undergoing autologous stem cell transplantation (Devine et al. 2004; Hübel et al. 2004). For a long time the mechanisms governing the progenitor cell release to the circulation remained poorly understood. Recent studies demonstrated that AMD3100 can accelerate progenitor cell mobilization through two different mechanisms. First, AMD3100 directly disrupts the CXCR4/CXCL12 interaction which is necessary for stem and progenitor cells homing and retention in the bone marrow and second, it might induce CXCL12 release from bone marrow stroma cells to the circulation that mediates progenitor cell mobilization in the peripheral blood (Dar et al. 2011). The observation that AMD3100 is highly specific for the CXCR4 receptor by inhibiting the binding of CXCL12 and the HIV cell entry with a high potency, led to the examination of its anti-cancer activity. The first studies in early 2000s demonstrated that AMD3100 inhibits survival, proliferation and migration of brain, breast, ovar-

ian cancer, and leukemia cells in response to CXCL12 stimulation (Cabioglu et al. 2005; Juarez et al. 2003; Rubin et al. 2003; Scotton et al. 2002; Smith et al. 2004; Tavor et al. 2004) that is attributable to the AMD3100-mediated inhibition of the extracellular signal pathways downstream of CXCR4 including phosphoinositide 3 kinase (PI3K)/AKT and human epidermal growth factor receptor 2 (HER2/neu)/c-Src kinase axis (Cabioglu et al. 2005; Rubin et al. 2003). Moreover, AMD3100 has been reported to render chemotherapeutic resistant myeloma, prostate and breast CSCs to chemotherapy and was further shown to prevent these cells from maintaining their tumor initiating and self-renewal properties (Ablett et al. 2013; Cabioglu et al. 2005; Dubrovska et al. 2012a, b; Gassenmaier et al. 2013; Gatti et al. 2013; Jung et al. 2012; Su et al. 2014). All these studies provided scientific rationale for clinical evaluation of AMD3100 in cancer treatment.

A number of clinical studies are underway testing an effect of AMD3100 in combination with conventional chemotherapy in patients with refractory acute myelogenous leukemia (NCT00512252), recurrent high-grade glioma (NCT01339039), relapsed and refractory multiple myeloma (NCT00903968), chronic lymphocytic leukemia and small lymphocytic lymphoma (NCT00694590), as well as relapsed and refractory hematologic malignancies in pediatric patients (NCT01319864). Combination of the conventional drugs such as etoposide, cyclophosphamide, mitoxantrone, cytarabine, bevacizumab and treatment with AMD3100 is used as a strategy to sensitize tumor cells to therapy and improve clinical outcomes. In addition, AMD3100 chemical scaffold was used for development more potent small molecule inhibitors of CXCR4 such as AMD3465, AMD11070 and MSX-122 (Hatse et al. 2005; Liang et al. 2012; Steen and Rosenkilde 2012). Like AMD3100, these inhibitors are blocking the cell surface binding of CXCL12 and are dose-dependently decreasing CXCR4/CXCL12-dependent cell proliferation, intracellular calcium signaling, migration, chemoresistance and tumorigenicity of various types of tumor cells including brain, breast, pancreatic, head and neck cancer, leukemia and melanoma (Liang et al. 2012, 2013; O'Boyle et al. 2013; Weekes et al. 2012). One of these new inhibitors, MSX-122, is in Phase I clinical trial for refractory metastatic or locally advanced solid tumors (NCT00591682), and another one, AMD11070 entered phase I clinical trials in HIV-infected individuals (NCT00361101, NCT00089466, NCT00063804).

Moreover, many efforts are underway to discover new small molecule CXCR4 antagonists based on the use of computational modeling (Veldkamp et al. 2010; Zhang et al. 2013a; Ziarek et al. 2012). Recent studies based on in silico screening have identified a new CXCR4 antagonist ICT5040 that specifically inhibits CXCL12-mediated cell migration and proliferation of glioblastoma cells (Vinader et al. 2013). Another study described an in silico screen, which has revealed a potent CXCR4 inhibitor 310454 efficiently abolishing CXCL12-mediated Ca^{2+} efflux in human monocytic cells (Veldkamp et al. 2010).

In addition to the development of the small molecule drugs, early work in the discovery of CXCR4 inhibitors was focused on the developing peptide analogues of the natural CXCR4 ligand that are able to selectively interact with the receptor and act as a competitive antagonist. Up to date, promising results were obtained using

CXCL12 analog CTCE-9908. This compound has demonstrated encouraging anti-tumor efficacy in preclinical studies. Studies employing human breast, esophageal, prostate cancer and osteosarcoma xenografts in mice and transgenic mouse models for breast cancer demonstrated that inhibition of CXCR4/CXCL12 chemokine pathway by using CTCE-9908 peptide significantly reduced the development of metastasis (Drenckhan et al. 2013; Hassan et al. 2011; Huang et al. 2009; Kim et al. 2008; Richert et al. 2009; B. Singh et al. 2010a; Wong et al. 2014). Treatment with CTCE-9908 also decreased primary tumor burden in mice bearing human breast, esophageal and prostate tumor xenografts (Drenckhan et al. 2013; Hassan et al. 2011; Huang et al. 2009; Porvasnik et al. 2009). The results of phase I/II clinical trial of CTCE-9908 in cancer patients with advanced metastatic disease showed that CTCE-9908 is well tolerated and has encouraging signs of anti-cancer efficacy (Hotte et al. 2007). Recent study of Gil et al. (2013) described novel tumor cell targeted therapy that delivered CTCE-9908 peptide via recombinant oncolytic vaccine virus (OVV). This OVV in based on the previously described VSC20 vaccinia virus that lack thymidine kinase (TK) gene and utilizes thymidine triphosphates for DNA synthesis from the nucleotide pool present in proliferating cells such as cancer cells. In addition, selective internalization and replication of this virus in cancer cells is also associated with cellular epidermal growth factor receptor (EGFR)/Rat sarcoma (Ras) pathway signaling and vascular endothelial growth factor (VEGF) derived from tumor cells. The vaccinia virus can be modified to deliver various growth factors, antigen and peptides that make recombinant virus a promising approach for gene therapy (Breitbach et al. 2011; Guo and Bartlett 2004; Hiley et al. 2013; McCart et al. 2001). Intravenous delivery of recombinant OVV expressing CTCE-9908 improves therapeutic outcome of the treatment a triple-negative 4T1 breast carcinoma in syngeneic mice compared to the soluble CTCE-9908. The enhanced antitumor effect of the virally delivered CTCE-9908 peptide was attributed to its high intra-tumor concentration compared to the soluble counterpart. Inhibition of tumor growth with the CTCE-9908 expressing virus was associated with an efficient destruction of tumor vasculature, decrease of CXCR4 and VEGF expression, reduction of intra-tumor endothelial and myeloid cells derived from bone marrow and induction of immune anti-tumor response (Gil et al. 2013). More recent study of the same group demonstrated that CTCE-9908-expressing OVV inhibits ovarian tumor growth in mouse xenograft models by decreasing immunosuppression and targeting ovarian CSCs (Gil et al. 2014). These findings demonstrate that engineering such of oncolytic virus armed with CXCR4 antagonists might pave the way to target multiple aspects of tumor biology on one hand, and on another hand, to decrease the toxicity of CXCR4 inhibition to the normal hematopoiesis due to the tropism of OVV to specifically target tumor cells.

Another peptide-based CXCR4 inhibitor, 4F-benzoyl-TN14003 (BKT140) is a chemically modified 14-residue polypeptide derived from a natural horseshoe crab protein (Tamamura et al. 1998, 2006). BKT140 is a highly selective CXCR4 antagonist which inhibits the growth of human chronic myelogenous leukemia, acute myeloid leukemia, multiple myeloma, non-Hodgkin lymphoma and non-small cell lung cancer xenografts in mice (Beider et al. 2013, 2011; Fahham et al. 2012), and

augments the effects of chemotherapeutic drugs and radiotherapy on tumor cell proliferation and tumor growth in xenograft mouse models (Beider et al. 2013, 2014; Fahham et al. 2012). Application of BKT140 in patients with multiple myeloma who were preparing for the autologous stem cell transplantation was associated with a favorable safety profile (Peled et al. 2014). Anti-tumor efficacy of this inhibitor has yet to be determined in further clinical studies.

In parallel, promising results were obtained with fully humanized antibody BMS-936564/MDX-1338 developed by Kuhne and co-workers (Kuhne et al. 2013). This antibody specifically recognizes CXCR4 receptor and blocks CXCL12 binding to CXCR4 expressing cells. BMS-936564 inhibited CXCL12-dependent cell migration, induced apoptosis on a panel of cancer cell lines and demonstrated a high antitumor activity when used as monotherapy to treat established xenograft tumors including acute myelogenous leukemia, non-Hodgkin lymphoma and multiple myeloma (Kuhne et al. 2013). Noteworthy, anti-CXCL12 antibody which was used in parallel with BMS-936564 for in vivo tumor treatment, did not inhibit tumor growth. It has been demonstrated that mechanism of cell apoptosis induced by anti-CXCR4 antibodies can be similar to the cell death induction by the binding of HIV-1 envelope glycoprotein gp120 to CXCR4 receptor (Berndt et al. 1998; Kuhne et al. 2013). Although BMS-936564 binds to the healthy peripheral blood cells, preliminary results of clinical trial for the treatment of leukemia and lymphoma patients demonstrated that this CXCR4 inhibition can be well tolerated (Kuhne et al. 2013) (NCT01120457).

Promising results obtained with BMS-936564 fostered other studies aiming to increase the efficacy of anti-CXCR4 antibodies by combining them with a cytotoxic drug. Kularatne and coworkers developed a chemically defined anti-CXCR4 antibody conjugated with anti-mitotic agent auristatin (Kularatne et al. 2014). This antibody conjugate was selectively toxic to CXCR4 expressing cells and significantly decreased tumor burden in the lung-seeding osteosarcoma xenograft tumor model.

Finally, recent observations demonstrated that activation of CXCR4 receptor depends on presentation of CXCL12 by cell surface glycosaminoglycans (GAG) (Allen et al. 2007; Friand et al. 2009; Hamel et al. 2009). Interaction of CXCL12 and other chemokines with GAG is proposed to be important for their function and stability, and targeting this interaction can be an effective approach in the cancer treatment (Shute 2012). The long-term use of hexuronic acid-based GAG, heparin in the treatment of cancer-associated thromboembolism pointed out its anti-cancer properties (Karamanos and Tzanakakis 2012; Kozlowski and Pavao 2011; Mousa and Petersen 2009). However, inhibition of the intracellular signaling by heparin is not specific. In addition, heparin is a complex charged oligosaccharide molecule, and from a therapeutic point of view it was reasonable to design less complex compounds that mimic the physiological role of heparin. Novel GAG mimetics bind to various chemokines and growth factors such as VEGF, heparin-binding EGF-like growth factor (HB-EGF), fibroblast growth factors FGF-1 and FGF-2, tumor necrosis factor α (TNF-α) and demonstrated strong anti-cancer activity associated with inhibition of cell proliferation, tumor angiogenesis and metastasis (Basappa et al. 2010; Ferro et al. 2007; Piccard et al. 2012; Zhao et al. 2006). Moreover, several

GAG mimetics that modulate CXCL12/CXCR4 axis have been tested in preclinical studies. Heparin and its mimetics block CXC12/CXCR4 signaling by binding to the receptor as well as to the ligand, thereby interfering with migration of various cell types, including cancer cells and bone-marrow derived mononuclear cells (Harvey et al. 2007; Seeger et al. 2012). Friand et al. demonstrated that chemically modified dextrans inhibit CXCL12-mediated hepatoma cell chemotaxis and anchorage-independent cell growth (Friand et al. 2009). Ma and co-workers have shown that low molecular weight heparin, enoxaparin, inhibited the CXCL12-stimulated colon cancer cell adhesion in vitro and suppressed formation of hepatic metastasis in xenograft mouse model (Ma et al. 2012). Another study demonstrated that butanoylated heparin derivative inhibited CXCR4 and CXCL12 expression, significantly reduced lung cancer cell proliferation in vitro and tumor growth in mice and rats (Yu et al. 2010). When given subcutaneously in mouse model of human breast cancer, heparin dodecasaccharides significantly inhibit tumor growth (Mellor et al. 2007). Interesting results were obtained by Patel and coworkers who performed a screening of GAG mimetic library for compounds which selectively inhibit the growth and self-renewal properties of colorectal CSCs (Patel et al. 2014). The GAGs, which inhibit spherogenic properties of colon cancer cells also significantly reduced expression of different CSC markers and self-renewal factors including CXCR4, CD44, CD133, LGR5, BMI1, OCT4, c-Myc and induced differentiation of colon CSCs. This data suggest that anti-cancer properties of some GAGs can be attributed to the targeting of CSC cells.

Although all above mentioned studies are encouraging, some important issues need to be considered prior to designing clinical trials that utilize CXCR4 inhibitors for cancer treatment. On one hand, multiple studies demonstrated that CXCL12/CXCR4 pathway antagonists inhibit the metastatic spread suggesting that CXCR4 inhibitors can be used for preventing metastatic dissemination and inhibiting the growth of metastatic lesions. On the other hand, CXCR4 antagonistic drugs given as a monotherapy proved their efficacy against the established tumors only for certain tumor entities. Thus, clinical translation of anti-CXCR4 therapy might require its combination with conventional therapy such as chemo- or radiotherapy, which shown promising results in most of preclinical studies (Duda et al. 2011). This increased efficacy of the combination therapy can be attributed to the activation of CXCR4 signaling pathway in response to the cellular stress including various therapies (Kioi et al. 2010; Kozin et al. 2010; Shaked et al. 2008; S. Singh et al. 2010b; Tabatabai et al. 2006). In turn, activation of CXCR4 axis contributes to tumor metastasis to lung and bone marrow which express a high level of CXCL12 and facilitates tumor neo-vascularization through mobilization of endothelial progenitor cells (Murakami et al. 2009). Therefore, combination of CXCR4 inhibition with other therapy might be more efficient in the inhibition of tumor growth and prevention tumor recurrence and metastatic spread compared to the current treatment protocols (Anna Dubrovska et al. 2012a; Murakami et al. 2009; Redjal et al. 2006). At the same time, therapeutic approach to prevent metastasis and tumor re-growth might require long-term administration of CXCR4 inhibitors that could potentially results in significant side effect because CXCR4 is expressed by numerous types of

healthy tissues. Moreover, it is unclear if prolonged effect of CXCR4 antagonists on bone marrow cell mobilization can be well tolerated. Development of novel CXCR4 inhibitors such as MSX-122 that does not possess stem cell mobilizing activity and tumor-specific OVV-based inhibitors could help to overcome some of these concerns (Liang et al. 2012). The ongoing clinical trials for CXCR4 inhibitors as chemosensitizers in patients with refractory and relapsed cancer as well as future clinical studies combining new generation CXCR4 antagonists and conventional therapy should address the questions about the effectiveness and safety on these therapeutic strategies.

6 Conclusion

Under physiological conditions CXCR4 is essential for bone marrow-specific homing and maintenance of circulating HSCs and for normal B-cell development. Tumor cells instead hijack the CXCR4/CXCL12 chemotaxis mechanisms, which allow them for homing to the bone marrow microenvironment. Moreover, this signaling is favoring their malignant growth and survival, provides anti-apoptotic signals and confers drug resistance. Several clinical trials established the CXCR4 expression as independent prognostic maker in AML, ALL and CLL. In addition, CXCR4 antagonists are promising agents, which inhibit the adhesion and migration of tumor cells to the protective bone marrow microenvironment. So far, the CXCR4-dependent mechanisms, which are able to explain minimal residual disease and subsequent relapse, are still unknown.

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Chapter 5
Non-coding RNAs in Cancer and Cancer Stem Cells

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Abstract Cancer stem cells (CSCs) have been identified in various types of human tumors. CSCs share a variety of signaling pathways with normal somatic stem cells, including those involved in self-renewal, differentiation, and the regulation of specific gene expression. Although the properties of CSCs, such as tumorigenicity and resistance to conventional therapeutics, have been the focus of intensive research in the field of cancer, the molecular mechanisms underlying the regulation of CSC properties remain incompletely understood. Therefore, many cancer researchers have investigated protein-coding genes and products, including surface markers that are involved in the acquisition of CSC properties. Recently, in addition to alterations in protein-coding genes, aberrant expression of non-coding RNAs such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) that play an important role in cellular, physiological, and developmental processes have been observed in various diseases including cancers. These non-coding RNAs also play important roles in the regulation of CSC properties. Several non-coding RNAs that regulate CSC properties have been identified; therefore, a better understanding of the mechanisms underlying such regulation could contribute to the identification of promising biomarkers and therapeutic targets. In this chapter, we discuss the general features of CSCs and the roles of non-coding RNAs, especially miRNAs and lncRNAs, in the regulation of CSC properties, and we summarize the current therapeutic strategies aimed at regulating non-coding RNAs for the purpose of CSC therapy.

Keywords Cancer stem cells • Non-coding RNAs • Long non-coding RNAs • MicroRNAs

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1 Cancer and Cancer Stem Cells

The cancer stem cell (CSC) concept is based on the hypothesis that cancer originates from a small population of tumor cells that exhibit the features of normal stem cells (Cohnheim 1875). Technological advances in cell-sorting techniques, single-cell analysis, and new animal models have supported the CSC concept (Lapidot et al. 1994; Bonnet and Dick 1997; Dalerba et al. 2011) and provided several lines of evidence that tumors are composed of heterogeneous cell populations, a feature that contributes to therapeutic resistance (Tam et al. 2013; Kobayashi et al. 2012). Moreover, clinical sequencing has also revealed that cancers are a heterogeneous mixture of genetically different subpopulations (Gerlinger et al. 2012). Therefore, CSCs are a major focus in current cancer research, not only for academic interest, but also for clinical practice, especially in regard to cancer drug discovery. In addition to genetic regulation of CSC properties, non-genetic determinants also play an important role in determining CSC properties related to epigenetic modifications such as DNA methylation or histone modification (Song et al. 2013b; Sakata-Yanagimoto et al. 2014). Recently, abnormalities in non-coding RNAs have been observed in various types of cancers (Tseng et al. 2014; Yuan et al. 2014; Valeri et al. 2014; Tazawa et al. 2007). Several studies have shown that non-coding RNAs play an important role in the generation of CSCs (Song et al. 2013b, a) and regulation for CSC properties (Chou et al. 2013; Deng et al. 2014; Zhang et al. 2014; Bu et al. 2013).

Several lines of evidence suggest that CSCs share a variety of biological properties with normal somatic stem cells, e.g., the capacity for self-renewal, propagation of differentiated progenitors, and expression of specific stem cell genes (Boumahdi et al. 2014). However, CSCs differ from normal stem cells in their resistance to chemotherapy, tumor formation capacity, and ability to metastasize (Todaro et al. 2014; Tam et al. 2013). In addition to several specific markers for CSCs (Oikawa et al. 2013; Wilson et al. 2014; Todaro et al. 2014; Bonnet and Dick 1997; Ginestier et al. 2007; Hermann et al. 2007; Li et al. 2007; Eramo et al. 2008; Yamashita et al. 2010; O'Brien et al. 2007; Ricci-Vitiani et al. 2007; Boiko et al. 2010; Pang et al. 2010; Singh et al. 2003, 2004; Haraguchi et al. 2010) (Table 5.1), glycosylation patterns also differ significantly between normal stem cells and CSCs (Karsten and Goletz 2013; Morgan et al. 2015; Liang et al. 2013).

Normal stem cells and CSCs share common signaling pathways such as Wnt, Notch, and Sonic Hedgehog that regulate induction of the epithelial-to-mesenchymal transition (EMT) and stem-like properties; dysregulation of these pathways is frequently observed in tumor initiation and development (Vermeulen et al. 2010; Chakrabarti et al. 2014). Chakrabarti et al. (2014) showed that the ΔNp63 isoform of the Trp63 transcription factor promotes normal and CSC activity by increasing the expression of the Wnt receptor Fzd7. In colon cancer, high activity of the Wnt pathway is associated with maintenance of CSC properties, and this activity is regulated by secreted factors such as hepatocyte growth factor derived from tumor-associated myofibroblasts (Vermeulen et al. 2010). The Notch pathway is also activated in breast, liver, and colon CSCs (Pan et al. 2014; Bu et al. 2013; Harrison et al. 2010). Alterations in Hedgehog signaling have been reported

Table 5.1 Representative markers for human cancer stem cells

Cancer	Cancer stem cell marker	Reference
AML	$CD34^+/CD38^-$	Bonnet and Dick 1997
Breast	$CD44^+/CD24^{-/low}$	Al-Hajj et al. 2003
	ALDH1 activity	Ginestier et al. 2007
Glioma	CD133	Singh et al. 2003, 2004
Colon	CD133	O'Brien et al. 2007; Ricci-Vitiani et al. 2007
	$CD44^+/EpCAM^+/CD166^+$	Dalerba et al. 2007
Metastatic Colon	$CD133^+/CD26^+$	Pang et al. 2010
	CD44v6	Todaro et al. 2014
Melanoma	CD271	Boiko et al. 2010
	ABCB5	Wilson et al. 2014
Pancreatic	$ESA^+/CD44^+/CD24^+$	Li et al. 2007
	CD47	Cioffi et al. 2015
Metastatic Pancreatic	$CD133^+/CXCR4^+$	Hermann et al. 2007
Lung	CD133	Eramo et al. 2008
Liver	$EpCAM^+/AFP^+$	Yamashita et al. 2010
	CD13	Haraguchi et al. 2010
	SALL4	Oikawa et al. 2013

AML acute myelogenous leukemia, *ALDH* aldehyde dehydrogenase, *EpCAM* epithelial cell adhesion molecule, *CXCR4* CXC chemokine receptor 4, *AFP* alpha-fetoprotein, *SALL4* Sal-like protein 4

in gastric, melanoma, glioblastoma and leukemic CSCs (Yoon et al. 2014; Santini et al. 2012; Hirata et al. 2014; Babashah et al. 2013).

Hippo signaling also plays important roles in the regulation of CSC properties. A number of studies have demonstrated that the Hippo pathway is involved in organ size control, stem cell maintenance, and tumor suppression (Aragona et al. 2013; Cordenonsi et al. 2011; Dong et al. 2007). The Hippo pathway, which is mediated by highly conserved serine/threonine kinases (MST1/2 and LATS1/2), negatively regulates the transcriptional co-activators YAP and TAZ (Piccolo et al. 2013). Cordenonsi et al. (2011) reported that TAZ activity is essential for the maintenance of self-renewal and tumor initiation capacities in breast CSCs (Cordenonsi et al. 2011). They also reported that TAZ is required for self-renewal of breast CSCs induced by EMT.

2 MicroRNAs

2.1 Biogenesis and Function of miRNAs

MicroRNAs (miRNAs), a class of small (21–25 nt) non-coding single-stranded RNAs, regulate gene expression at the post-transcriptional level by binding to the 3′-untranslated regions (3′UTRs) or open reading frames of target mRNAs, leading to their degradation or translational repression (Garofalo and Croce 2011; Takahashi

et al. 2014). MiRNAs are primarily transcribed by RNA polymerase II as long primary transcripts called primary miRNAs (pri-miRNAs). In the nucleus, pri-miRNA transcripts are converted into 60–100 nt precursor miRNAs (pre-miRNAs) by Drosha and its co-factor DiGeorge critical region 8 (DGCR8) (Fig. 5.1) (Han et al. 2004; Gregory et al. 2004). Drosha is a member of the RNase III family that cleaves pri-miRNAs to release pre-miRNAs (Han et al. 2004). The *DGCR8* gene is located in chromosomal region 22q11.2, and its heterozygous deletion causes DiGeorge syndrome (Shiohama et al. 2003). *DGCR8* can stabilize Drosha through physical

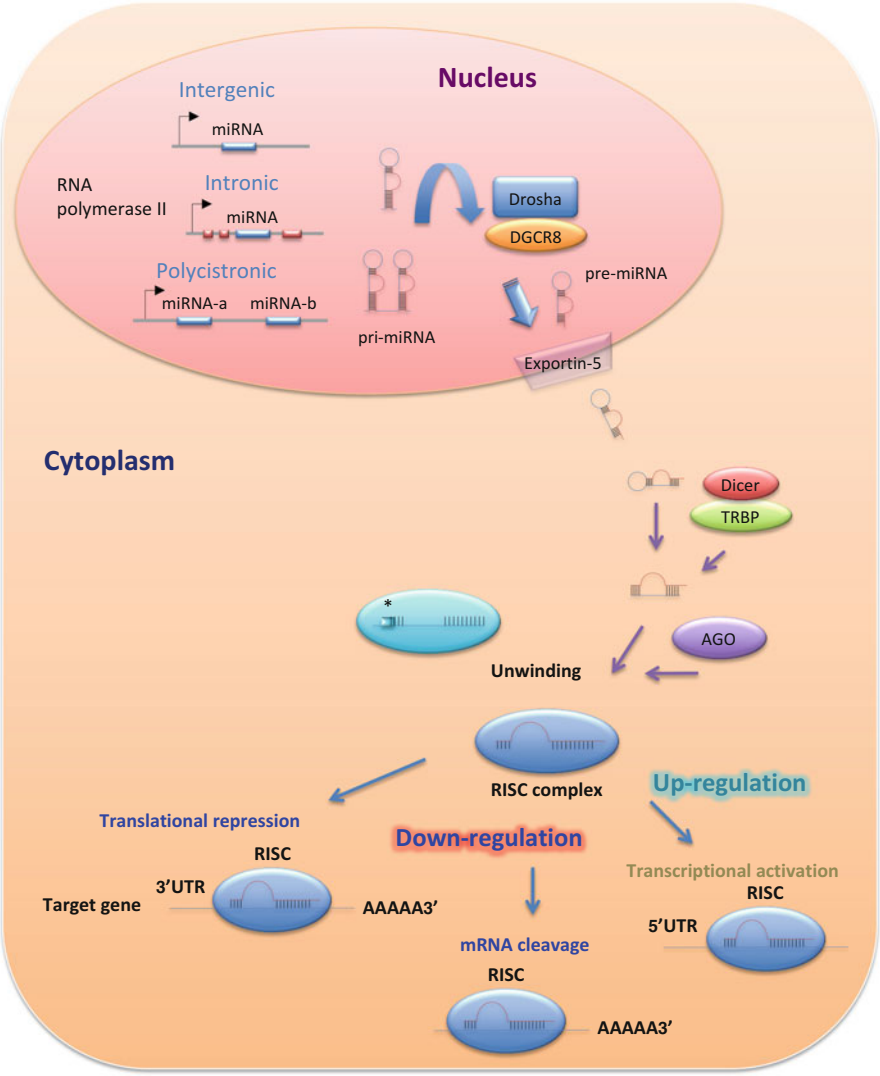

Fig. 5.1 MicroRNA biogenesis, processing and function. The biogenesis of miRNAs begins in the nucleus and is completed in the cytoplasm. For more details, see the text

interactions and is essential for miRNA maturation (Yeom et al. 2006). Recently, Cheng et al. reported that methyl-CpG binding protein 2 (MECP2), which is associated with severe neurodevelopmental disorders such as Rett syndrome (Lewis et al. 1992), inhibits nuclear miRNA processing and neural development by interfering with assembly of Drosha–DGCR8 complex (Cheng et al. 2014). Pre-miRNAs, the product of pri-miRNA cleavage, are exported to the cytoplasm from nucleus by Exportin-5 and Ran-GTP complex (Lund et al. 2004; Yi et al. 2003), and then further cleaved into a miRNA:miRNA* complex by the RNase III Dicer, which can associate with two different double-stranded RNA (dsRNA)-binding proteins, protein activator of PKR (PACT) and trans-activation response RNA-binding protein (TRBP). Recently, the nuclear export of pre-miRNAs is also induced after DNA damage in an Ataxia Telangiectasia Mutated (ATM)-dependent manner (Wan et al. 2013). Lee et al. (2013) also reported a functional difference between PACT and TRBP in miRNA and small interfering RNA (siRNA) biogenesis. In Dicer-mediated small RNA processing, PACT in complex with Dicer exhibited lower levels of siRNA processing activity than a TRBP-Dicer complex.

One of the two strands works as a guide strand, whereas its counterpart (miRNA*) is usually subjected to degradation (Iorio and Croce 2012). However, recent studies report that miRNA* can function as a guide strand and may play an important role in gene regulation (Luo et al. 2014; Josson et al. 2014). The mature miRNA is incorporated into the RNA-induced silencing complex, which contains the GW182 and Argonaute proteins. As a component of this complex, the mature miRNA represses gene expression by binding to partially complementary sequences in the miRNA response elements of its target mRNAs (Iorio and Croce 2012).

2.2 MiRNA-Mediated Gene Regulation

Several studies report that miRNAs positively regulate gene expression by associating with the promoter elements or 5′UTR of their targets (Liu et al. 2013; Place et al. 2008). Place et al. (2008) reported that miR-373 induces the up-regulation of E-cadherin and cold-shock domain-containing protein C2 (CSDC2) by binding to its target site on both promoters. Liu et al. also reported that miR-483-5p, encoded in the insulin-like growth factor 2 (*IGF2*) gene, directly binds to the 5′UTR of this gene and induces *IGF2* expression in human fetal kidney and Wilms' tumors by promoting the interaction between RNA helicase and IGF2 transcripts (Liu et al. 2013).

2.3 MiRNAs and Cancer

MiRNAs play important roles in the initiation and progression of human cancer, and expression profiling of miRNAs in human malignancies has revealed signatures associated with tumor development and progression (Babashah 2014; Volinia and Croce 2013; Zhang et al. 2013a). Chromosomal regions encoding oncogenic

miRNAs that induce the repression of a tumor suppressor gene can be amplified in association with tumor malignancy. This amplification would result in up-regulation of oncogenic miRNAs and down-regulation of tumor suppressor genes. On the other hand, miRNAs that suppress oncogenes are often located at chromosomal fragile sites, where deletions can occur, leading to reduction or loss of miRNAs and overexpression of their target oncogenes. Dysregulation of miRNA expression affects several aspects of cancer progression such as the enhancement of anti-apoptotic activity, therapeutic resistance, tissue invasion, and metastasis (Babashah and Soleimani 2011; Shah et al. 2014; Havelange et al. 2014; Martello et al. 2010). Recent evidence suggests that miRNAs are also involved in tumor initiation through the regulation of CSC properties such as asymmetric cell division, tumor seeding ability, and chemoresistance (Hwang et al. 2014; Bu et al. 2013; Yu et al. 2007).

3 Long Non-coding RNAs in Cancer and Cancer Stem Cells

3.1 Long Non-coding RNAs

Non-coding RNAs other than miRNAs are also involved in diverse biological processes, including tumor malignancy (Tseng et al. 2014). In this respect, long non-coding RNAs (lncRNAs), defined as a non-coding RNAs longer than 200 nucleotides that are not translated into proteins (Ling et al. 2013), represent potential targets for cancer therapy. LncRNAs are frequently transcribed by RNA polymerase II and located between protein-coding regions. LncRNAs are identified using histone marker signatures associated with RNA polymerase II, specifically by the trimethylation of lysine 4 and lysine 36 of histone 3 (H3K4me3 and H3K36me3) (Guttman et al. 2009; Khalil et al. 2009). LncRNAs are also found in an antisense orientation to protein-coding genes (Bertozzi et al. 2011; Vigetti et al. 2014).

According to their functions and structures, lncRNAs are classified into subgroups such as circular RNAs (Zhang et al. 2013b), natural antisense transcripts (Rinn et al. 2007), transcribed ultraconserved regions (T-UCRs) (Nielsen et al. 2014), long enhancer ncRNAs (Nielsen et al. 2014), and long intergenic ncRNAs (lincRNAs) (Khalil et al. 2009). Several studies demonstrated that lncRNAs are involved in epigenetic changes through association with chromatin-modifying complexes. Khalil et al. (2009) reported that about 38 % of lncRNAs in various types of cells bind to chromatin-modifying complexes.

3.2 LncRNAs and Cancer

LncRNAs play diverse roles in tumor development in many types of cancers (Table 5.2). One well-characterized function of lncRNA is the recruitment of epigenetic modifiers to specific loci, leading to alterations in chromatin state. For example,

Table 5.2 LncRNAs and cancer

LncRNA	Function	Reference
HOTAIR	Retargeting of the PRC2 and H3K27me3 patterns	Gupta et al. 2010
FAL1	Protein interaction with BMI1	Hu et al. 2014a
LincRNA-p21	Repression of p53 downstream genes through the physical interaction with heterogeneous nuclear ribonucleoprotein K (HNRNPK)	Huarte et al. 2010
LUNAR1	Up-regulation of IGFR1 mRNA expression	Trimarchi et al. 2014
lncRNA-ATB	- Up-regulation of EMT regulators via competitively suppressing miR-200 family - Activation of STAT3 signaling via promoting IL-11 mRNA stability	Yuan et al. 2014
MALAT1	Regulation of alternative splicing via modulating the distribution and activity of pre-mRNA splicing factors	Tripathi et al. 2010
INXS	Induction of pro-apoptotic BCL-XS via physical interaction with splicing modulator, Sam68	Paronetto et al. 2007
PTENP1	An miRNA decoy: Competitive inhibition of PTEN targeting miRNAs	Poliseno et al. 2010

HOX antisense intergenic RNA (*HOTAIR*), which is a 2.2 kb lncRNA (Rinn et al. 2007), promotes breast cancer metastasis through genome-wide retargeting of the Polycomb repressive complex 2 (PRC2) and H3K27me3 patterns (Gupta et al. 2010). Recently, focally amplified lncRNA on chromosome 1 (*FAL1*) was identified as an oncogenic lncRNA in several types of cancer. *FAL1* regulates the stability of the epigenetic repressor BMI1 to modulate transcription of a number of genes including CDKN1A (p21), resulting in tumor growth (Hu et al. 2014a).

LncRNAs also play an important role in tumor development through transcriptional activation and repression. *LincRNA-p21* is a p53-activated lncRNA that acts as a repressor in p53-dependent transcriptional responses (Huarte et al. 2010). In response to DNA damage, *LincRNA-p21* induces the transcriptional repression of p53 downstream genes through a physical interaction with heterogeneous nuclear ribonucleoprotein K, which leads to p53-dependent apoptotic induction. In human T-cell acute lymphoblastic leukemia, the Notch-regulated lncRNA *LUNAR1* promotes tumor malignancy by up-regulating IGFR1 mRNA expression and maintaining IGFR1 signaling (Trimarchi et al. 2014). Trimarchi et al. (2014) reported that *LUNAR1* serves as a scaffold to modulate the activity of Mediator and RNA polymerase II at the *IGF1R* enhancer site.

Another important function of lncRNA is post-transcriptional modification. LncRNA activated by TGF-β (*lncRNA-ATB*) is associated with metastasis of hepatocellular carcinoma (HCC) and correlated with poor prognosis. *LncRNA-ATB* induces up-regulation of EMT regulators (ZEB1 and ZEB2) by competitively binding the miR-200 family that suppresses ZEB1 and ZEB2 expression (Yuan et al. 2014). Yuan et al. (2014) also found that *lncRNA-ATB* promotes HCC cell

colonization at sites of metastasis by binding IL-11 mRNA and increasing IL-11mRNA stability, leading to activation of STAT3 signaling.

Some lncRNAs are regulators of RNA processing (DeOcesano-Pereira et al. 2014; Ji et al. 2003). *MALAT1* (Metastasis-associated lung adenocarcinoma transcript 1) is a lncRNA of more than 8000 nucleotides detected in non-small cell lung cancer (NSCLC). Elevated expression of *MALAT1* is significantly associated with NSCLC metastasis (Ji et al. 2003). *MALAT1*, which is stably localized in the nucleus, regulates alternative splicing by modulating the distribution and activity of pre-mRNA splicing factors (Tripathi et al. 2010). *MALAT1* interacts with serine/arginine proteins and influences the distribution of these and other splicing factors in nuclear speckle domains where pre-mRNA splicing factors are localized (Hutchinson et al. 2007; Tripathi et al. 2010). A recent study showed that *MALAT1* promotes metastasis of lung cancer cells through up-regulation of genes associated with metastasis (Gutschner et al. 2013). These reports suggest that *MALAT1* regulates gene expression by two distinct mechanisms. INXS (Intronic BCL-XS-inducing lncRNA) was also identified as a lncRNA that modulates alternative splicing in cancer cells (DeOcesano-Pereira et al. 2014). *INXS* is transcribed from the genomic strand opposite to BCL-XL. Compared to normal tissues, expression of *INXS* is quite low in tumor tissues such as kidney and prostate tumor tissues. Alternative splicing of the BCL-X mRNA generates two products, pro-apoptotic BCL-XS and anti-apoptotic BCL-XL, and the splicing pattern of the BCL-X gene is regulated by the splicing modulator Sam68 (Paronetto et al. 2007). DeOcesano-Pereira et al. (2014) found that *INXS* promotes generation of the BCL-XS isoform via a physical interaction with Sam68.

Several studies reported that lncRNAs also act as decoys and inhibit miRNA function in cancer cells (Hu et al. 2014b; Poliseno et al. 2010). Poliseno et al. (2010) reported a functional relationship between the tumor suppressor gene *PTEN* and its pseudogene *PTENP1*. Although *PTENP1* has a missense mutation in the initiator methionine codon that prevents its translation, *PTENP1* contains a 3′UTR that is 1 kilobase shorter than that of *PTEN*. The *PTENP1* 3′UTR contains a conserved region with high homology to *PTEN* 3′UTR. Therefore, *PTENP1* is a direct target of *PTEN*-targeting miRNAs such as miR-19b, miR-20a, and miR-21 and acts as a miRNA decoy for *PTEN*. Hu et al. (2014b) also found that lncRNA termed as *GAPLINC* is highly expressed in gastric cancer tissues and regulates CD44 expression as a miRNA decoy for miR-211-3p which targets CD44 and *GAPLINC*. *GAPLINC* inhibits the miR-211-3p–mediated suppression of CD44, and its aberrant expression is associated with poor prognosis in gastric cancer patients.

Taken together, these observations demonstrate that lncRNAs play important roles in various aspects of tumor development through multiple mechanisms, described above. Therefore, a deeper understanding of lncRNAs will reveal their roles in cancer biology and open new therapeutic avenues for cancer treatment.

Table 5.3 LncRNAs and cancer stem cells

LncRNA	Function	Reference
GAPLINC	Inhibition of the miR-211-3p-mediated suppression of CD44	Hu et al. 2014b
HOTAIR	EMT induction by suppressing the HoxD10-meditaed gene regulation	Matsui et al. 2010; Zhang et al. 2014
linc-ROR	EMT induction by competitively inhibiting miR-205 function	Hou et al. 2014
Xist	The expression of Xist is inversely correlated with tumor suppressive function of HDAC inhibitor	Salvador et al. 2013

3.3 LncRNAs and CSCs

LncRNAs are also involved in the regulation of CSC properties (Table 5.3). Padua Alves et al. (2013) reported that the HOTAIR expression was up-regulated by TGF-β1, which resulted in the acquisition of colony-forming ability and EMT induction in colon and breast cancer cells. They also found that *HOTAIR* was highly expressed in $CD133^{+}/CD44^{+}$ cells in colon cancer cell lines. Zhang et al. reported that *HOTAIR* promotes the acquisition of CSC properties by suppressing HoxD10-meditaed gene regulation in breast cancer cells (Zhang et al. 2014). MiR-7, one of the transcriptional targets of HoxD10, inhibits the EMT phenotype through the direct target of SET domain bifurcated 1 (SETDB1), which is important for the maintenance of the stem cell state (Matsui et al. 2010; Zhang et al. 2014).

The EMT phenotype is also regulated by *linc-ROR*, the first lncRNA identified in induced pluripotent stem cells (iPSCs) (Hou et al. 2014; Wang et al. 2013). Wang et al. (2013) reported that *linc-ROR* functions as a decoy for miR-145 that targets the key pluripotency factors Oct4, Sox2, and Nanog. In breast cancer, *linc-ROR* also competitively inhibits miR-205 that targets the EMT regulator ZEB2.

Recently, Salvador et al. (2013) found that the lncRNA*Xist* (X-inactive specific transcript) might be a biomarker that predicts the response of breast cancer cells to Histone deacetylase inhibitors (HDACi) that induce the differentiation of CSCs into non-CSCs. Consistent with this idea, in breast cancer cells that express low levels of Xist, low concentrations of HDACi suppress tumor seeding ability.

Therefore, these findings suggest that lncRNAs play a functional role in the generation and regulation of CSCs through the competitive inhibition of tumor-suppressive miRNAs. Furthermore, as with miRNAs, profiling of lncRNA expression can be used to evaluate the therapeutic response to cancer treatment.

4 Role of MicroRNAs in the Regulation of Cancer Stem Cell Properties

A number of studies demonstrate that miRNA exerts a functional role in CSC biology in various types of cancer cells. In this section, we present current findings about the roles of miRNAs in the regulation of the key biological properties of CSCs.

4.1 Leukemia Stem Cells

Using an integrated approach that combines miRNA expression analysis and bioinformatics tools to predict miRNA targets, distinct miRNAs were identified as a fine tuner for each step of hematopoiesis, including the reconstitution potential of hematopoietic stem cells (Arnold et al. 2011).

The miR-17-92 cluster was identified as oncogenic based on the observation that it promotes the formation of Myc-driven B-cell lymphomas in a mouse model (He et al. 2005). Li et al. (2014) reported that Myc maintains a neoplastic state via miR-17-92–mediated suppression of chromatin regulatory genes such as *Sin3b* and *Btg1*, as well as the apoptosis regulator gene, *Bim*.

Elevated expression of miR-155 in early B-cells leads to polyclonal expansion of pre-leukemic B-cells (Costinean et al. 2006). Wang et al. (2014a) revealed a biological link between miR-155 and Notch signaling in the development of myeloproliferative disorders. Notch signaling suppresses miR-155 expression by promoting binding of RBPJ to the miR-155 promoter. Therefore, loss of Notch signaling in bone marrow induces the up-regulation of miR-155 in bone marrow endothelial cells, resulting in suppression of the nuclear factor kB inhibitor (NF- kB inhibitor), kB-Ras1 that inhibits proinflammatory cytokine production.

Dysregulation of single miRNAs can contribute to hematological malignancies, including AML and myelodysplastic syndrome (de Leeuw et al. 2014; Song et al. 2013a; Lechman et al. 2012). Lechman et al. (2012) reported that miR-126 is highly expressed in hematopoietic stem cells (HSCs) and early progenitors and plays an important role in determining HSC pool size. Importantly, they also demonstrated that knockdown of miR-126 induced HSC proliferation without exhaustion. de Leeuw et al. (2014) also found that miR-126 is highly expressed in HSCs and AML relative to leukemic progenitors, and that attenuating miR-126 preferentially decreases the number AML cells without affecting the survival of bone marrow cells. These reports suggest that miR-126 is a promising target for the treatment of AML. MiR-22 has been associated with myelodysplastic syndrome and hematological malignancies (Song et al. 2013a). This miRNA increases the methylation of ten-eleven-translocation gene 2 tumor suppressor (TET2) target genes such as *AIM2* and *SP140* via direct targeting of *TET2*, thereby promoting hematopoietic stem cell self-renewal and transformation.

4.2 Breast CSCs

In 2003, CSCs of solid tumors were first identified and isolated from breast tumors. Al-Hajj et al. (2003) reported a $CD44^{+}/CD24^{-/low}$ cell population with high tumor-initiating capacity. Subsequently, Mani et al. (2008) also found that a $CD44^{+}/CD24^{-/low}$ cell population is generated from a $CD44^{low}/CD24^{high}$ cell population upon EMT induction.

The EMT is an important evolutionarily conserved process that occurs during embryonic development in many species of mammals (Lee et al. 2006). Because the EMT program is often activated during tumor invasion and metastasis, the molecular mechanisms and biological process underlying the acquisition of invasive and metastatic ability by cancer cells have been the subjects of intensive research. Mani et al. reported that human breast cancer cells induced to undergo EMT by expression of EMT regulators such as Snail and Twist exhibit a $CD44^{+}/CD24^{-/low}$ antigen phenotype and high tumorigenicity (Mani et al. 2008).

A molecular link between the EMT and microRNA has been demonstrated in breast cancer (Gregory et al. 2008; Burk et al. 2008). Expression of the miR-200 family strongly inhibits the EMT phenotype induced by TGF-β through a direct target of the ZEB family of transcription factors that tightly regulates the EMT (Gregory et al. 2008). The miR-200 family is composed of five miRNAs classified into two clusters: miR-200a, miR-200b, and miR-429 on human chromosome 1; and miR-200c and miR-141 on human chromosome 12 (Gregory et al. 2008). Burk et al. (2008) also identified reciprocal repression between members of the miR-200 family and ZEB1 in several types of cancer cells. ZEB1 directly suppresses the transcription of miR-141 and miR-200c in pancreatic, colorectal, and breast cancer cells.

The expression level of miR-200c is also transcriptionally regulated by p53 in breast cancer cells (Chang et al. 2011). p53 up-regulates the expression of miR-200c by directly binding to the promoter region of miR-200c. Thus, p53 plays a critical role in suppressing both EMT and EMT-associated CSC phenotypes via transcriptional activation of miR-200c expression in breast cancer cells.

Normal and cancer human mammary epithelial cells can be isolated and characterized on the basis of their ALDH activities (Ginestier et al. 2007). Using this approach, Ibara et al. (Ibarra et al. 2007) demonstrated elevated expression of miR-205 and miR-22 in mouse mammary progenitor cells. MiR-22 functions as an epigenetic modifier that promotes EMT and metastasis in breast cancer by directly suppressing enzymes of the TET family, which are involved in DNA demethylation (Song et al. 2013b). Because the TET family promotes the demethylation of the miR-200 promoter, miR-22 promotes CSC properties such as EMT and a metastatic phenotype through suppression of the miR-200 family. Therefore, Song et al. reported the first evidence that chromatin-remodeling systems associated with cell fate (self-renewal versus differentiation) are regulated by opposing sets of miRNAs.

4.3 Brain CSCs

In brain tumors, CD133 was first used for the identification of CSCs (Singh et al. 2003). CD133, also known as Promini-1, is a pentaspan membrane glycoprotein first identified as a surface marker of HSCs. CD133 is highly expressed on CD34-positive hematopoietic stem and progenitor cells derived from human fetal liver,

bone marrow, and blood (Yin et al. 1997). Recently, Brescia et al. (2013) reported the functional role of CD133 in the maintenance of glioblastoma (GBM) stem cells using tumor cells derived from GBM patients. CD133 silencing inhibited tumor growth and self-renewal capacity of CD133-expressing GBM. On the other hand, several studies reported that CD133-negative cells derived from CD133-positive cells also have tumor-initiating capacity in GBM and colon cancer (Son et al. 2009; Shmelkov et al. 2008). Hence, further investigations are required to identify the molecules involved in targeting CSCs and the non-CSCs that differentiate from CSCs.

Stage-specific embryonic antigen 1 (SSEA-1) was also identified as a CSC marker in GBM (Son et al. 2009). SSEA-1 is expressed in tumorigenic $CD133^-$ GBM cells, suggesting that SSEA-1 is a more general CSC marker in GBM. In $CD133^+$ or SSEA-1^+ GBM cells, OCT4 and SOX2 transcriptionally promote the up-regulation of a DNA methyltransferase (*DNMT*) gene that induces promoter methylation of multiple miRNAs including miR-148a. MiR-148a inhibits the formation of GBM neurospheres in vitro and GBM tumor seeding ability in vivo in animal models (Lopez-Bertoni et al. 2014). Therefore, OCT4 and SOX2 support the CSC properties in GBM through the DNMT-mediated suppression of miR-148a.

In GBM, migration and invasion of CSCs are also regulated by miRNAs (Wang et al. 2014c). miR-20a and 106a (miR-20a/106a) promote the invasiveness of $CD133^+$ CSCs in GBM (Wang et al. 2014c). MiR-20a/106a promote CSC invasion by directly targeting tissue inhibitor of metalloproteinases-2 (TIMP-2).

4.4 Colon CSCs

CD133 was initially used for identification and isolation of colon CSCs (O'Brien et al. 2007; Ricci-Vitiani et al. 2007). After these findings, CD44, epithelial surface antigen (EpCAM), and CD166 were identified as alternative colon CSC markers (Dalerba et al. 2007). In addition to these established CSC markers, the expression of leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5), identified as a stem cell marker in small intestine and colon (Barker et al. 2007), was also observed in colon CSCs (Vermeulen et al. 2008).

MiR-34a plays an important role in determining the cell division patterns of colon CSCs (Bu et al. 2013); specifically, this miRNA inhibits the asymmetric cell division of colon CSCs and promotes the differentiation of CSCs by suppressing Notch activity. Because Notch signaling is dysregulated directly by epigenetic and genetic modifications and indirectly by interaction with the Wnt pathway (Fre et al. 2009; Taketo 2011), activation of Notch signaling is frequently observed in colon cancer cells. In addition, Notch signaling is required for tumor seeding ability and self-renewal activity of colon CSCs (Sikandar et al. 2010). Thus, in colon CSCs, miR-34a inhibits Notch signaling via a direct target of Notch1 receptor (Bu et al. 2013; Li et al. 2009), resulting in the differentiation of CSCs into non-CSCs.

MiR-146 is also involved in the regulation of asymmetric cell division of colon CSCs (Hwang et al. 2014). In colon CSCs, miR-146a supports the self-renewal and tumor seeding ability via direct targeting of Numb that inhibits β-catenin expression, Wnt activity, and asymmetric cell division. Hwang et al. (2014) also found that Snail, an EMT regulator, activates *miR-146a* transcription by promoting the formation of a complex containing β-catenin and TCF4. Therefore, in Snail highly expressing colon CSCs, miR-146a plays a critical role in the regulation of CSC properties.

5 Therapeutic Approaches to Target Cancer Stem Cells

To improve conventional therapies for intractable cancer, therapies that target CSCs are urgently required. Because CSCs are molecularly different from non-CSCs and the majority of tumor cells, it is necessary to identify target molecules or small compounds that contribute to elimination or reduction of the CSC population. Gupta et al. (2009) identified salinomycin as a selective inhibitor of breast CSCs using a library of 16,000 natural and commercial chemical small compounds. Because salinomycin is a polyether antibiotic produced by a strain of *Streptomyces albus* with activity against Gram-positive bacteria (Miyazaki et al. 1974), it is used in chicken fodder as an anti-coccidial drug (Danforth et al. 1977). Gupta et al. (2009) found that salinomycin is capable of selectively killing breast CSCs. Although the molecular mechanisms for the elimination of CSCs by salinomycin have not been fully elucidated, several studies have reported the mechanisms and pharmacological action of salinomycin in human CSCs (Fuchs et al. 2010; Lu et al. 2011; Tang et al. 2011).

Several dietary compounds can also directly or indirectly affect the properties of CSCs and induce the elimination or differentiation of breast CSCs (Wang et al. 2014b; Kaushik et al. 2014; Hagiwara et al. 2012). Therefore, natural dietary compounds have attracted increasing attention from cancer researchers. Resveratrol is a non-toxic natural product that was first isolated from the roots of white hellebore and later from *Polygonum cupsidatum*, a medicinal plant (Aggarwal et al. 2004). In recent years, resveratrol has become widely consumed as a nutritional supplement (Prasad 2012), and its multifaceted biological effects are associated with anti-mutagenic and anti-cancer properties (Patel et al. 2013; Hagiwara et al. 2012). Hagiwara et al. (2012) reported that resveratrol supports the function of miRNAs via up-regulation of Ago2 expression in breast cancer cells, leading to the suppression of CSC properties.

Metformin also selectively reduces the proportion of CSCs in breast cancer cells (Hirsch et al. 2009). Metformin (1,1-dimethylbiguanide), a biguanide derivative originally isolated from French lilac, was used as a botanic medicine for polyuria in medieval Europe (Quinn et al. 2013). Polyuria is frequently observed in diabetes patients. Hirsch et al. (2013) reported that metformin selectively inhibits cellular transformation and the proliferation of CSCs by suppressing the nuclear transloca-

tion of NF-kB and phosphorylation of STAT3 in CSCs. Furthermore, metformin suppresses the growth of breast cancer cells by modulating DICER and c-Myc (Blandino et al. 2012). Because metformin promotes the expression of some specific miRNAs by up-regulating *DICER* expression, it might exert multifaceted biological effects against a variety of diseases through miRNAs and their targets. In light of these results, the identification of non-toxic natural compounds capable of suppressing the properties of CSCs through the regulation of miRNA expression represents a promising approach to overcoming intractable cancers.

6 Conclusions

As a consequence of technological developments such as severe immunodeficient mice (NOD/SCID and NOG mice) and quantitative PCR coupled with single-cell sorting, the heterogeneity of tumor tissues and plasticity of cancer cells (i.e., the ability to convert from a non-CSC to a CSC phenotype) have become important issues in cancer research. Therefore, clinical oncologists and cancer researchers need to consider and identify which cancer cells are involved in tumor seeding and progression, including chemotherapy resistance and metastasis. Because non-coding RNAs such as miRNAs and lncRNAs can function as tumor suppressors or oncogenes and play important roles in the generation and regulation of CSCs, non-coding RNAs are considered to be functional markers of CSCs. Therefore, a more detailed understanding of the functions of these non-coding RNAs in CSC biology might improve the treatment of cancer patients and lead to the development of clinical applications for cancer diagnosis, treatment and prognosis.

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Chapter 6
Self-Renewal Pathways in Mammary Stem Cells and Carcinogenesis

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Abstract Accumulating evidence shows the presence of a subpopulation of cancer stem cells (CSCs) in many cancers including breast cancer. The breast cancer stem cells (BCSC) are resistant to traditional treatments and able to initiate tumorigenesis, suggesting that they may contribute to therapy resistance and relapse. The expression of specific markers in BCSCs and development of mouse model has facilitated the study and several intrinsic and extrinsic pathways maintaining BCSC population have been exploited. Several signal transduction pathways such as Wnt, Notch, Hedgehog, Bmi-1, PI3K/AKT and IL6 are known to regulate self-renewal pathways in normal stem cells; while in CSCs these pathways are normally dysregulated due to accumulated mutations and epigenetic changes. Understanding the signaling pathways through which CSCs regulate their self-renewal and maintenance, and hence tumor growth and metastasis is important for developing targeted therapies to abrogate CSCs.

Keywords Mammary stem cells • Breast cancer stem cells • Carcinogenesis • Self-renewal pathways

1 Introduction

The mammary gland is a dynamic organ which undergoes massive morphological changes during puberty, pregnancy, lactation and involution. Under the influence of systemic sex hormones including estrogen and progesterone, the mammary epithelium proliferate and differentiate to accumulate fat and develop lobulo-alveolar structure and lactating ducts during puberty, pregnancy and lactation, and undergo regression with massive apoptosis during mammary involution. The dynamic cycle

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of mammary gland suggests the existence of stem-like cells within mammary epithelium (Hennighausen and Robinson 2005; Smalley and Ashworth 2003). Recent prospective isolation and characterization of mammary stem cells and progenitor populations in the mouse (Asselin-Labat et al. 2006; Stingl et al. 2006; Shackleton et al. 2006; Sleeman et al. 2005) and human (Shipitsin et al. 2007; Villadsen et al. 2007; Lim et al. 2009; Eirew et al. 2008; Keller et al. 2012) mammary gland provide further evidence that the mammary epithelium is organized in a hierarchical manner, and that a single mammary stem cell (MaSC), which resides in the basal/myoepithelial layer, can functionally reconstitute a mammary gland by giving rise to differentiated progenies through various lineage-restricted progenitor cells (Stingl et al. 2006; Shackleton et al. 2006; Visvader 2009). Therefore, abnormalities of normal development and proliferation are not surprisingly seen in breast tissue related to the frequent proliferation/involution cellular events.

There is also increasing evidence that stem cells might be the targets of transformation during carcinogenesis. Breast cancer is defined as a malignant tumor arising from uncontrolled proliferating breast epithelial cells, and results in lumps or thickening of the breast tissue. Breast cancer is highly heterogeneous; the heterogeneity of breast cancers is manifested by their classification into a number of distinct subtypes, each with a characteristic transcriptome and molecular expression signature (Visvader 2009). The categorization into different types helps clinicians to choose the most effective therapies. For example, estrogen and progesterone receptors (ER and PR) are important prognostic indicators in breast cancer; tumors with these two receptors tend to be well differentiated and responsive to hormone-therapies, and such patients tend to have a prolonged disease-free survival rate. Tamoxifen that inhibits cell proliferation by competing with endogenous estrogens on the ER site is the most frequent prescription medicine for ER positive breast cancer patients. Although early detection and development in adjuvant systemic chemotherapy and radio-therapy increase relapse-free and overall survival rates, development of therapy-resistance and disease recurrence remain problematic. These features are key characteristics of the cancer stem cell (CSC) hypothesis: the existence of a group of cancer cells able to survive conventional therapies and give rise to tumor growth later. Accumulating evidence suggests the existence of subpopulations within a tumor that are resistant to treatments and display “stem cell” properties (Wicha et al. 2006; Liu and Wicha 2010; Charafe-Jauffret et al. 2008).

A unique property of stem cells is their ability to undergo self-renewal divisions. In normal organogenesis this process is tightly regulated. The deregulation of self-renewal might be one of the key events involved in carcinogenesis. Indeed, pathways involving cell signaling pathways and transcription factors involved in the self-renewal of normal stem cells have all been implicated in carcinogenesis. These pathways include Hedgehog, Notch and Wnt, the transcription factor B lymphoma Mo-MLV insertion region 1 (Bmi-1), PI3K/AKT, as well as IL6 pathway. In this article we review evidence that these pathways are involved in both stem cell self-renewal and carcinogenesis, which provides support for the concept that breast carcinogenesis results from the deregulation of self-renewal pathways of normal mammary stem cells. We also highlight the potential therapeutic approaches and clinical implications of targeting these signaling pathways for breast cancer treatment.

2 Mammary Stem Cells Versus Breast Cancer Stem Cells

Stem cells are defined by their ability to self-renew and differentiate into multiple lineages. They are important for embryonic development and tissue regeneration in adults. In adult tissues, stem cells sit in specific niches, which are important in stem cell regulation and maintenance by responding to intrinsic and extrinsic signals. In human mammary gland, the presence of breast epithelial stem cells were initially described by their ability to form colonies, which morphology resemble myoepithelial or luminal phenotypes or express markers exclusive for these two populations (Stingl et al. 1998). Mammary epithelial stem cells exhibit the following properties:

1. Expression of distinct proteins has been used to identify stem cell populations for a long time, although the functions of these markers are not always well understood. In mammary epithelial cells, MUC-1 glycoprotein $(MUC\text{-}1)^{+}$/common acute lymphoblastic leukemia antigen $(CALLA)^{-}$/epithelial-specific antigen $(ESA)^{+}$ and the $MUC\text{-}1^{-\text{ to }+/-}/CALLA^{+/-\text{ to }+}/ESA^{+}$ were suggested as the progenitor markers for ductal and alveolar cells by the group that first identified human breast epithelial progenitor (Stingl et al. 1998). Recent studies show that the use of cell surface markers CD49f (α6 integrin) and CD29 (β1 intergrin) together with CD24 (heat stable antigen) or EpCAM (epithelial specific antigen), have been shown to enrich for mammary stem/progenitors in the mouse and human mammary gland (Stingl et al. 2006; Shackleton et al. 2006).
2. The ability to eject fluorescent dyes such as Hoechst due to high activity of several transmembrane transporters leads to formation of a side population (SP). Research on normal mammary epithelium found limited SP cells, but these cells were able to differentiate into ductal and lobular cells both in vitro and in vivo (Alvi et al. 2003), indicating their stem/progenitor property.
3. The ability to display aldehyde dehydrogenase activity, which can be assessed by the ALDEFLUOR assay via flow cytometry. In vivo study showed only $Aldefluor^{+}$ cells of normal mammary epithelium were able to repopulate fat pad in mouse but not the $Aldefluor^{+}$ population. And the structures formed by $Aldefluor^{+}$ mammary epithelial cells resembled the human mammary duct phenotype as well as expressed same pattern of cytokeratins (Ginestier et al. 2007).
4. The potential to survive and proliferate when grown in anchorage-independent environment with the presence of growth factors in the form of spheroids. These floating 3D structures, termed mammospheres were capable to differentiate into both epithelial and myoepithelial lineages. More importantly, this culture technique maintained the self-renewal and multilineage potential of the mammary epithelial stem/progenitor populations (Dontu et al. 2003).

Taken together, the establishment of biomarkers, in vitro and in vivo models from studies of normal mammary stem cells has facilitated the isolation and characterization of such cells in malignant breasts.

The concept of stem driven carcinogenesis was first proposed in 1855 by The German pathologist Rudolf Virchow. For years, direct evidence to prove cancer

stem cell existence is not found. In 1997, cancer stem cell (CSCs) was first identified in acute myeloid leukemia when specific cell surface protein markers became available for distinguishing a rare population of cells (Bonnet and Dick 1997). Since then, researchers have shown many human cancers, including breast cancer, might have a population of cells that display stem cell properties (Wicha et al. 2006; Liu and Wicha 2010; Charafe-Jauffret et al. 2008). These properties include self-renewal, which gives rise to tumorigenesis, and differentiation, which contributes to cancer cell heterogeneity. These cells may mediate metastasis and, by virtue of their relative resistance to chemotherapy and radiation, contribute to treatment relapse following therapy.

Flow cytometry utilizing cell surface markers is one of useful ways to identify putative breast cancer stem cells (BCSCs). Our laboratory first isolated breast cancer initiating cells based on the expression of three unique cell surface antigens, which are epithelial specific antigen (ESA) and CD44 but not CD24. $ESA^+CD44^+CD24^-$ cells were capable to generate tumors when as few as 200 cells were injected into mammary fat pad of NOD/SCID mice, whereas cells without these markers isolated from the same tumors did not, even 100-fold more cells were injected (Al-Hajj et al. 2003). Subsequent studies show $CD44^+CD24^-$ can define a population enriched in BCSCs. The $CD44^+CD24^-$ BCSCs possess the ability to self-renew and to differentiate which undergoes xenograft mammary tumor formation and progression. Other cell surface markers, like CD49f and CD133, can be used to identify BCSCs in different breast cancer subtypes combining with $CD44^+CD24^-$. In breast cancer, elevated CD49f expression is associated with reduced survival (Friedrichs et al. 1995) and knockdown of its partner CD104 decreases in vivo tumorigenicity (Lipscomb et al. 2005). The cells enriched in $CD44^+CD49f^{hi}CD133^{hi}$ subset displays heightened tumorigenicity and self-renewal in vivo, and the capacity to give rise to functional and molecular heterogeneity (Meyer et al. 2010).

BCSCs can also be isolated or studied using the Aldefluor assay based on aldehyde dehydrogenase (ALDH) activity (Ginestier et al. 2007). ALDH is an enzyme responsible for the oxidation of intracellular aldehydes, it plays an important role in stem cell differentiation via retinoic acid metabolism. The commercially available Aldefluor kit (StemCell Technologies, Inc., Vancouver, British Columbia, Canada) contains a BODIPY-aminoacetaldehyde (BAAA) substrate labeled with a fluorochrome that is converted into BODIPY-aminoacetate (BAA) by ALDH catabolism. Cells expressing high ALDH activity have brighter fluorescence and can be indentified using DEAB (an inhibitor of the enzymatic reaction) as the isotype control for FACS analysis. The combination of Aldefluor positivity with other unique stem cell surface markers such as $CD133^+$ and $CD24^-CD44^+$ has been shown to further label and locate BCSCs (Charafe-Jauffret et al. 2009).

Furthermore, in primary breast xenografts, $CD44^+CD24^-$ and ALDH identified overlapping, but non-identical cell populations, each capable of initiating tumors in NOD/SCID mice. Tumor cells that expressed both CSC markers (i.e. $CD44^+CD24^-$ and $ALDH^+$) displayed the greatest tumor-initiating capacity, generating tumors in

NOD/SCID mice from as few as 20 cells. The $EpCAM^{+}CD24^{-}CD44^{+}$ and $ALDH^{+}$ populations across different subtypes of breast cancers identify anatomically distinct BCSCs with respective EMT (epithelial-to-mesenchymal transition) and MET (mesenchymal-to-epithelial transition) gene expression profiles, and they dynamically transit between the mesenchymal and the epithelial states reflective of their normal counterparts in the mammary epithelial hierarchy (Liu et al. 2014).

BCSCs can subsequently be sorted and assayed for clonogenic potential in vitro and tumorigenicity in vivo by xenotransplantation using immune-compromised mice. The latter is the gold standard for assessing BCSC activity. Cells isolated from tumorspheres exhibit multi-lineage differentiation potential when given serum and extracellular matrix such as collagen (Dontu et al. 2003). Another use is based on the ability of stem cells to exclude DNA dye such as Hoechst 33,342 by membrane transporters, and the SP has been shown to contain the most tumorigenic population within breast cancer cell line when injected in vivo (Dontu et al. 2003; Hadnagy et al. 2006). A more recent method to characterize CSCs in vitro is the cell membrane label-retaining assay. This assay uses the PKH fluorescent dye series, which consist of a fluorophore attached to a peptide backbone that irreversibly binds to the lipid bilayer of cell membranes. The use of PKH dye label-retaining mammosphere assay has recently been used to identify both normal MaSCs and BCSCs (Pece et al. 2010; Cicalese et al. 2009).

3 Self-Renewal Signaling Pathways

Several pathways such as Hedgehog, Notch, and Wnt and a transcription factor Bmi-1 are known to regulate self-renewal pathways in normal stem cells; while in CSCs these pathways are normally dysregulated due to accumulated mutations and epigenetic changes. Conventional cancer therapies normally target aberrant pathways in the rapid proliferating bulk tumor cells, but often spare the CSCs leading to tumor recurrence and metastasis. Therefore, the design of new therapies must be based on targeting the signaling pathways that affect both CSCs as well as bulk tumor cells. We review the role of these signaling pathways in stem cell self-renewal as well as evidence that deregulation of these pathways is important in mammary carcinogenesis. And we review the main pathways that are involved in CSC self-renewal along with their potential therapeutic implications.

3.1 Hedgehog Signaling

The hedgehog signaling pathway was first identified in Drosophila, where it is required for early embryo patterning. Recent studies show that Hh signaling pathway regulates cell proliferation, cell fate determination and stem/progenitor cell

maintenance (Cohen 2003; Lewis and Veltmaat 2004). Three hedgehog ligands have been identified in mammals: Sonic Hedgehog (Shh), Desert Hedgehog (Dhh), and Indian Hedgehog (Ihh), all of which are secreted glycoproteins. After secretion, these ligands bind to the hedgehog-interacting protein 1 (Hip1) and Patched (Ptch) to activate Gli transcription factors. In the absence of ligands, two transmembrane proteins, Ptch and Smoothened (Smo), form the receptor complex. Ptch binds to Smo and blocks its function. This inhibition is relieved in the presence of ligands, and Smo initiates a signaling cascade that results in the release of transcription factors Glis from cytoplasmic proteins fused (Fu) and suppressor of fused (SuFu). In the inactive situation, SuFu prevents Glis from translocating to the nucleus; in the active situation, Fu inhibits SuFu and Glis are released. Smo interacts in a signaling cascade that results in activation of the transcription factors. Gli proteins, include Gli1, Gli2, and Gli3, in turn translocate into the nucleus and control target gene transcription (di Magliano and Hebrok 2003). Gli regulates the transcription of several genes, including those controlling cell proliferation such as cyclin D, cyclin E, Myc, components of the epidermal growth factor pathway, and angiogenesis components including platelet derived-growth factor and vascular endothelial growth factor.

Ptch1, Gli1 and Gli2 genes are expressed in normal human mammary stem/progenitor cells cultured as mammospheres and are down-regulated during differentiation. Overexpression of Ptch1, Gli1 and Gli2 has been shown in $CD24^{-}CD44^{+}$ BCSCs compared to non-stem cells. Activation of Hh signaling using Hh ligand or Gli1/Gli2 overexpression increases mammosphere formation, mammosphere size and multi-lineage progenitors, whereas inhibition of the pathway via cyclopamine results in a reduction of tumorigenic potential. Moreover, overexpression of Gli2 in human mammary stem/progenitor cells enriched in mammosphere culture produces ductal hyperplasias when these cells are implanted into the humanized fatpads of NOD-SCID mice (Liu et al. 2006).

The Hh pathway was targeted using cyclopamine, a steroidal alkaloid that down-regulates Gli1 by binding to Smo and hence suppresses the growth of breast cancer cells (Kubo et al. 2004). Subsequently, new Hh inhibitors have been developed by chemically modifying cyclopamine (Tremblay et al. 2008). At present, GDC-0449 (Vismodegib, trade name: Erivedge), the first Hh pathway inhibitor approved by FDA (Robarge et al. 2009), is undergoing clinical trials in combination with the Notch signaling inhibitor RO4929097 (a gamma-secretase inhibitor, GSI) for metastatic breast cancers where tumors cannot be surgically removed, but this trial has been suspended owing to side effects associated with this therapy, and other combination therapies are currently undergoing (http://clinicaltrials.gov/) (Hui et al. 2013). It would be particularly interesting to see the effect of these Hh inhibitors on CSCs as the Hh pathway may be activated in CSCs in response to chemotherapy or during recurrence. Since Hh signaling also imparts chemoresistance (Olive et al. 2009), the most effective cancer therapy would likely include Hh inhibitors along with cytotoxic chemotherapy.

3.2 Notch Signaling

Notch transmembrane receptors are part of signaling pathways that are crucial in the regulation of the fate of cells in a variety of tissues. The Notch proteins, involves four homologous trans-membrane receptors, Notch 1 to Notch 4, are expressed in a variety of stem or early progenitor cells. Upon binding to their cognate ligands (DSL ligands: Delta, Delta like, Jagged1 and Jagged2), the intracellular domain (ICD) of Notch is cleaved and translocates into the nucleus to activate its target genes (Chiba 2006). This process is activated by serial cleavage events involving members of the ADAM (for 'a disintegrin and metalloproteinase') protease family, as well as an intramembrane cleavage regulated by γ–secretase (presenilin). Notch signaling has emerged as a key regulator involving stem cell maintenance, cell-fate specification, and differentiation (Chiba 2006) and dysregulated Notch signaling has been implicated in a number of human malignancies (Roy et al. 2007; Radtke and Raj 2003). In vitro, overexpression of the constitutively active form of Notch4 inhibits the differentiation of normal breast epithelial cells. In vivo, Notch4 has an important role both in normal mammary development and in carcinogenesis. Transgenic mice harboring a constitutively active Notch4 under the regulation of mouse mammary tumor virus promoter exhibited arrested mammary gland development, and eventually developed poorly differentiated adenocarcinomas. Knockdown of the canonical Notch effector Cbf-1 in MaSC-enriched population was found to increase stem cell activity whereas constitutive Notch signaling specifically targeted luminal progenitor cells for expansion, leading to hyperplasia and tumorigenesis (Bouras et al. 2008). These findings about the role of Notch in promoting the self-renewal of mammary stem cells, in addition to previous observations that it can function as a proto-oncogene (Uyttendaele et al. 1998; Soriano et al. 2000), suggest that abnormal Notch signaling might be involved in carcinogenesis, through the deregulation of normal mammary stem cell self-renewal. In human breast cancers, co-expression of Jag1 and Notch1 is associated with poor overall survival (Reedijk et al. 2005). In $ESA^{+}CD24^{-}CD44^{+}$ BCSCs, Notch-4 and Notch-1 activity was found to be eightfold and fourfold higher respectively compared to the differentiated bulk tumor cells (Harrison et al. 2010). Pharmacologic or genetic inhibition of Notch1 or Notch4 reduced stem cell activity in vitro and reduced tumor formation in vivo (Harrison et al. 2010). Elevated Notch-1 signaling also contributes to drug resistance as down-regulation of Notch-1 signaling in human breast cancer cells increases chemo-sensitivity to doxorubicin and docetaxel (Zang et al. 2010).

Several important oncogenic pathways such as ErbB2, Jak/Stat, TGF-β, NF-κB, Wnt and Hedgehog interact with the Notch pathway (Olsauskas-Kuprys et al. 2013). For example, ErbB2 has been shown to induce Notch-1 activity through Cyclin D1 induction (Lindsay et al. 2008). Combined treatment of DAPT, a Notch inhibitor with ErbB2 inhibitor Lapatinib effectively targets stem/progenitor cells both in vitro and in vivo in breast ductal carcinoma in situ (DCIS) (Farnie et al. 2013). Another study showed that Notch-1 signaling is decreased in ErbB-2 overexpressing SKBR3,

BT474 and MCF7/HER2 cells and that HER2-targeted therapies using trastuzumb or lapatinib reactivated Notch-1 and rendered them sensitive to GSIs (Osipo et al. 2008). These studies suggest that combined treatment of GSI with HER2 targeted therapies may be more beneficial and could potentially reverse the resistance of HER2 targeted therapies especially in CSCs. It has also shown the association between Notch3 and EGFR receptors. Inhibition of EGFR kinase activity leads to activation of Notch transcriptional targets in a gamma secretase inhibitor sensitive manner and causes Notch activation, leading to an increase in ALDH+ cells (Arasada et al. 2014). Together these studies suggest that treatments aimed at molecules that affect multiple stem cell pathways could present a novel strategy for targeted therapies.

3.3 Wnt Signaling

The Wnt pathway regulates cell fate determination in a number of tissues, including the mammary gland. The Wnts are a family of secreted glycoproteins. The well-characterized Wnt signaling pathway is called the canonical Wnt pathway, in which Wnt ligands signal through the stabilization of β-catenin. Several β-catenin-independent Wnt signaling pathways, known as non-canonical, have been shown to be crucial for different aspects of vertebrate embryo development (Veeman et al. 2003). In the canonical Wnt pathway, Wnt proteins bind to a family of Frizzled receptors in a complex with the low-density lipoprotein receptor-related proteins 5 and 6 (LRP5 or LRP6). Activation of these receptors results in the accumulation of intracellular β-catenin. In the absence of Wnt signaling, β-catenin remains in the cytoplasm, where it forms a complex with other proteins, including the tumor suppressor adenomatous polyposis coli and axin, and well as glycogen synthase kinase (GSK)-3β. GSK-3β is able to phosphorylate β-catenin leading to its ubiquitin-mediated degradation. When the Wnt pathway is activated, GSK-3β is inhibited, blocking β-catenin phosphorylation. Activation of the Wnt pathway phosphorylates GSK3β and hence stabilizes β-catenin which then translocates to the nucleus, where it binds to and activates the transcription factors T-cell factor/lymphoid enhancer factor (TCF/LEF), which then activates several oncogenes such as ID2, MMP7, and c-Myc (Klaus and Birchmeier 2008). The noncanonical Wnt signaling pathway is known to act through Rho family small GTPase, calcium and protein kinase A signaling. It involves Frizzled receptors and the proteoglycan co-receptor Knypek. A cytoplasmic signal transduction protein Dishevelled (Dsh) localizes to the cell membrane through its DEP domain. Dsh activates Rho through the bridging molecule Daam1. Dsh can also stimulate calcium flux and sequentially activates the calcium-sensitive kinases protein kinase C and calmodulin-dependent protein kinase II (Veeman et al. 2003).

The Wnt pathway regulates cell fate determination in several tissues including the mammary gland (Logan and Nusse 2004). In LRP5 knockout mammary glands, very few stem or progenitor cells were present compared to wild type mammary

glands (Badders et al. 2009). Activation of Wnt signaling and its components have been implicated in variety of cancers including breast (Bafico et al. 2004; Klopocki et al. 2004; Nagahata et al. 2003; Nakopoulou et al. 2006). Transgenic mice overexpressing Wnt-1 in mammary glands were enriched for epithelial cells expressing progenitor cell markers keratin 6 and Sca1 and tumors that developed in these mice contained cells expressing keratin 6 (Li et al. 2003). This suggests that mammary stem cells and/or progenitors may be the targets for oncogenesis by Wnt pathway. Furthermore, the transforming activity of Wnt effectors was shown to be correlated with their ability to induce accumulation of mammary progenitor cells (Liu et al. 2004). The AKT/β-catenin pathway is also activated by anti-angiogenic agents such as sunitinib and bevacizumab which drives CSCs expansion through HIF1 alpha (Conley et al. 2012). Targeting of the Wnt pathway could be achieved by several approaches. For example, methylation-associated silencing of SFRP1 was shown to inhibit Wnt signaling in breast cancer (Yang et al. 2009). In breast cancer cell lines including MCF7, HuL100 and SKBR3, incubation with Wnt1 monoclonal antibody has been used to inhibit Wnt-1 signaling and induce apoptosis (He et al. 2004). The redundancy between different ligands may suggest that antibody directed Wnt inhibition would not be a successful approach, but some cancers have been shown to rely heavily on specific Wnt isoforms, it may be a viable approach in those cancers. For tumors which do not rely on specific Wnt, the use of pan-Wnt inhibitor may be more efficacious. A recent study demonstrated that a soluble ligand binding domain of Fzd8, Fzd8-CRD-Fc, inhibited autocrine Wnt signaling in vitro, as well as in multiple xenograft models (DeAlmeida et al. 2007).

3.4 Bmi-1 Signaling

Bmi-1 is a transcriptional repressor belonging to the polycomb group (PcG) of transcription factors. It was first identified in a B-cell lymphoma (Alkema et al. 1993). Bmi-1 has been shown to be a key regulator of the self-renewal of many normal and cancer stem cells. Recent studies have shown that Bmi-1 is a marker of CSCs. Bmi-1 is found to be overexpressed in several human breast cancer cell lines, playing a pivotal role in maintaining stem cells phenotype and carcinogenesis. Several recent studies have demonstrated a role of Bmi-1 in regulating EMT and migration of breast cancer cells. Bmi-1 is overexpressed in primary human breast cancer and metastatic breast cancer cells, regulating EMT and metastasis of cancer cells (Li et al. 2014). In Twist-induced EMT, Twist1 and Bmi-1 act cooperatively to repress expression of epithelial marker, E-cadherin, and promote tumor-initiating capability (Yang et al. 2010). Overexpressed of Bmi-1 due to positive feedback loop between Bmi-1 and Wnt is likely to increase the fraction of CSCs and endow tumors resistance to drug (Cho et al. 2013).

Since its essential role in cancer cell and contribute to drug resistance, Bmi-1 might be a new target in breast cancer therapy. Researchers have found that elevated Bmi-1 expression is correlated with advanced stage of breast cancer, especially

basal-like breast cancer (Guo et al. 2010; Wang et al. 2012). Drugs aimed at Bmi-1 or its regulation pathway may be potent in controlling or even completely removing cancer. Several attempts have been made. Joon-Ho et al. suggests that since Akt phosphorylating Bmi-1 inhibiting self-renewal of hemopoietic stem cells, overexpression of Akt may reduce the fraction of CSCs (Liu et al. 2012). Kreso and colleague found that, PTC-209, a Bmi-1 inhibitor, can effectively block self-renewal of cancer initiating cells in vitro and tumor growth in mouse xenograft in colon cancer (Kreso et al. 2014). Besides, there is report about Bmi-1 autoantibody, pointing out that Bmi-1 autoantibody acting as a new potential biomarker for cervical carcinoma, implying an antibody therapy (Tong et al. 2011). Yet whether this compound and antibody is also apply to breast cancer, more studies need to be explored.

3.5 PI3K/AKT Signaling

The PI3K/Akt signaling pathway plays a pivotal role in cell survival, proliferation, migration, metabolism, angiogenesis, and apoptosis. Akt kinase is activated after activation of PI3K in growth factor receptor-mediated signaling cascades. A simple model for activation by growth factor is that, upon growth factor combining to receptor tyrosine kinase, PI3K is recruited to the plasma membrane via its Src-homology (SH) domain. Catalytic subunit of PI3K then phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) and thus generates phosphatidylinositol-3,4,5-trisphosphate (PIP3). The increased PIP3 acts as an anchor point and recruits PH-domain-containing protein, such as Akt. The recruited Akt is then phosphorylated at Thr-308 and Ser-473 by phosphoinositide-dependent kinase 1 (PDK1) and PDK2, respectively, and then phosphorylates downstream effectors in cytoplasm and nuclear (Franke 2008).

The PI3K/Akt signaling pathway participates in a wide spectrum of tumor types. The aberrant activation of the PI3K/Akt pathway and mutations in the phosphatase and tensin homolog (PTEN) gene, whose product inhibits downstream products of PI3K and activation of Akt, have been validated by epidemiolodical and experimental studies as a key step in the initiation and maintenance of human cancers. Study has found that PTEN/PI3K/Akt pathway plays an essential role in maintenance prostate cancer stem-like cells (CSLCs). A $CD133^{+}/CD44^{+}$ population of cells enriched in the prostate cancer progenitor cells have the potential to initiate tumor. These cells preferentially activate PI3K/Akt pathway in the sphere-forming condition. Inhibition of PI3K can repress the growth of these cells. What's more, shRNA-mediated knockdown of PTEN increases the sphere-forming, clonogenic and tumorigenic potential (Dubrovska et al. 2009). Study in colitis indicated that, PI3K/Akt signaling cooperates with the Wnt to increase activation of β-catenin via phosphorylating on Ser-552, thus promoting activation of progenitor cells in the process from chronic ulcerative colitis to colitis-associated cancer (Lee et al. 2010). Anne and colleagues suggested that PTEN/PI3K/Akt may have a role in regulating mouse and human gliomas, for the observation that Akt can regulate the activity of ABCG2,

which endows CSLCs with chemoresistance, and that knockdown of PTEN can increase the fraction of CSLCs (Bleau et al. 2009).

The alteration of PI3K/Akt signaling pathway, through either activation of oncogenes or inactivation of tumor suppressors, is also a commonly disrupted pathway in human breast cancer and has a major role in anti-cancer drug resistance. Zhou et al. observed that in MCF7 cell line, PI3K/PTEN signaling has a pivotal role in maintaining the survival and proliferation of side population cells that are rare cell populations known to enrich CSLCs within cancers and cell lines (Zhou et al. 2007). There is study suggests that, different PI3K/Akt pathway aberrations may play distinct role in the pathogenesis of different breast cancer subtypes. PI3K mutations are more common in hormone receptor-positive and HER2-positive than in basal-like tumors, while Akt and PTEN mutations are restricted to hormone receptor-positive tumors. Besides, PI3K and PTEN mutations are more common in cell lines than in tumors, while Akt mutations are absent in cell lines. Thus PI3K-targeted therapy in hormone receptor-positive breast cancer might be a potential therapy (Stemke-Hale et al. 2008). On the other hand, Serra et al. found that, in HER2-overexpressing breast cancer cells, inhibition of PI3K can abolish Akt activation but result in a compensatory activation of the ERK signaling pathway due to activation of HER family receptors, which can be prevented by either MEK inhibitor or anti-HER2 monoclonal antibodies and tyrosine kinase inhibitors. Therefore they proposed a combined therapy administrating PI3K inhibitors with either HER2 or MEK inhibitors (Serra et al. 2011). But whether these therapies is of use in clinical and is there any other interaction between PI3K/Akt and other pathway, much more need to be done.

3.6 IL6 Signaling

Cytokines generated by cells within the tumor microenvironment stimulate CSC self-renewal, which then may promote tumor growth and metastasis (Sansone et al. 2007; Ginestier et al. 2010). IL6, one of the Cytokines, plays a crucial role in the pathophysiology of cancer (Rose-John et al. 2006). In cancer patients, high levels of IL6 are associated with poor patient outcome and in pre-clinical models IL6 has been shown to promote tumorigenesis, angiogenesis and metastasis (Scheller and Rose-John 2006; Fisman and Tenenbaum 2010). IL6 triggers the gp130 and IL6R proteins to form a complex after IL6 interacting with its receptor, IL6R, and this complexes could activate Stat3 (Heinrich et al. 1998). Stat3 activation in turn leads to transcriptional activation of NF-κB in inflammatory cells which secrete additional IL6 and IL-8 acting on tumor cells. Thus, these cytokines generate a positive feedback loop between immune cells and tumor cells which further stimulates the tumor stem cell components accelerating metastasis and therapeutic resistance. Utilizing mouse xenografts, we have demonstrated that bone marrow mesenchymal stem cells are recruited to sites of growing breast cancers by gradients if IL6 (Liu et al. 2011). IL6 could interact with a lot of factors to affect the cancer. IL6 regulated

the transcriptional and epigenetic mechanisms of CYP2E1 and CYP1B1 in colorectal cancer to promote colorectal carcinogenesis (Patel et al. 2014). In pancreatic carcinoma, heparanase induced macrophages to produce more IL6 to induce STAT3 signaling and to augment pancreatic carcinoma cell proliferation (Hermano et al. 2014). Maria Ouzounova et al. has confirmed the relationship between IL6 and p53/PTEN (Ouzounova et al. 2014). They developed transformed MCF10A model by simultaneous knockdown of p53 and PTEN and in this model, they demonstrated that enhanced the expression of SOCS3 could reduce the tumor growth and inhibited metastasis. Importantly, SOCS3 negatively regulated the IL6/Stat3/NF-kB pathway and this is why it could have the effect on tumor. All of above suggested that IL6 plays critical role in cancers and IL6 has been used as a therapeutic target. Some data suggest that in ER+ breast cancer the patients have "high-producer" IL6 genotypes and poor prognosis. And those tumor cells had a high expression of IL6 gp130 receptor, JAK/STAT signaling and cyclin D, suggesting targets for intervention in these patients (Demichele et al. 2014). Clinical trials utilizing IL6 blocking antibodies have been initiated for the treatment of multiple myeloma with early encouraging results (Fulciniti et al. 2009). Furthermore, anti-IL6R antibody, tocilizumab, has been approved for the treatment of arthritis (Ohsugi and Kishimoto 2008) with little clinical toxicity.

4 Interaction Between Self-Renewal Pathways

The signaling pathways regulating stem cell self-renewal described above have interactions between each other in vivo. Accumulating evidences have showed the interactions between the Hedgehog signaling and the Notch signaling pathways. Notch, executing the cell fate, can be reinforced by the secretion of Shh (López et al. 2003). In order to examine the relationship between Hedge signaling and Notch signaling, we utilized mammosphere-derived culture systems, and it was found that when we activated the Notch pathway, the hedgehog pathway was subsequently activated and the expression of Ptch and Gli was also up-regulated. Additionally, if we blocked activation of Notch signaling by γ-secretase, the hedgehog pathway remained unactivated (Liu et al. 2006). However, studies have shown that during arterial endothelial differentiation, Shh, one of the hedgehog ligands, acts upstream of Notch to determine arterial cell fate (Lawson et al. 2002). As Shh activates hedgehog pathway, expression of HES1, the Notch pathway target in the mammospheres, is also up-regulated, which could be blocked by the hedgehog inhibitor cyclopamine (Liu et al. 2006). So, it might be a feedback loop that Hedgehog and Notch forms to regulate normal and cancer development.

Studies have shown that Wnt pathways are involved in the regulation of multiple pathways. β-catenin and LEF-1 are the two markers of active Wnt signaling.

Evidences have shown that in the skin, the activation of β-catenin and LEF-1 correlates with Notch-dependent transformation (Kopper and Hajdú 2004). In chronic myeloid leukemia, Activation of Stat3 inducing by hyperactive Shh signaling in CD34+ CML up-regulates expression of downstream target genes Wnt3a, Lef1, CyclinD1, Gli1 and p21, leading to overactive Wnt signaling. The hyperactive Wnt in turn together with Shh promotes the expression of Lef1 and CyclinD1, causing uncontrolling proliferation of cells (Sengupta et al. 2007). Recent studies represent that the Wnt pathway interacts with Notch through Wnt/TCF target Jagged-1, a Notch ligand, and Mel-18, a negative regulator of Bmi-1. Knockdown of Mel-18 has been shown to enhance the self-renewal of BCSCs whereas its overexpression inhibited the number and self-renewal activity of BCSCs. Mel-18 blockade up-regulated Jagged-1 expression and consequently activated the Notch pathway (Won et al. 2012). Activation of Wnt signaling and its components have been implicated in variety of cancers including breast cancer, indicating cancer arise and develop through interactions of different pathways.

Evidences have shown that Bmi-1 acts as a downstream target in Shh pathway, as its expression rapidly increased after addition of Shh or overexpression of the Shh target Gli in cerebellar granular cells. Due to the association between overexpression of Bmi-1 and overexpression of Ptch and Sufu, the Hedgehog pathway is at least partially activated in Bmi-1 overexpression tumors (Leung et al. 2004). Our studies indicate that activation of Hedgehog pathway and Notch pathway resulted in the expression of Bmi-1 in the mammosphere culture system, and this induced expression could be aborted using inhibitors targeting Hedgehog and Notch (Liu et al. 2006). Recent studies have explored the relationship between Bmi-1 and Wnt signaling pathway. Joon-Ho et al. demonstrates a positive feedback loop regulating the autoregulation of Bmi-1. Bmi-1 can transcriptionally repress DKK family protein, a Wnt inhibitor, and up-regulate the Wnt factors, hence up-regulating the canonical Wnt signaling pathway, resulting up-regulation of c-Myc, which in turn promotes up-regulation of Bmi-1 (Cho et al. 2013).

Furthermore, recent study demonstrated that IL6 treatment triggered Notch-3–dependent up-regulation of the Notch ligand Jagged-1 and promotion of MS and MCF-7–derived spheroid growth (Sansone et al. 2007). And some papers indicated that IL6 meditated Jagged1-Notch1 promotes breast cancer bone metastasis (Sethi et al. 2011). All of those suggests that IL6 may regulate stem cells through Notch pathways. On the contrary, it has been reported that suppression of IL6, GM-CSF, and MMP-3 production by DLL-1 blockade might be responsible for the amelioration of arthritis in a mouse model of RA (Sekine et al. 2014). What's more, ADAM10 mediates a canonical Notch-dependent regulation of IL6 through Dll4 in human endothelial cells (Pabois et al. 2014). Thus, IL6 and Notch could interact with each other to regulate CSC.

Together, all these studies demonstrate extensive interaction between the signaling pathways that regulate stem cell self-renewal as elucidated in Fig. 6.1.

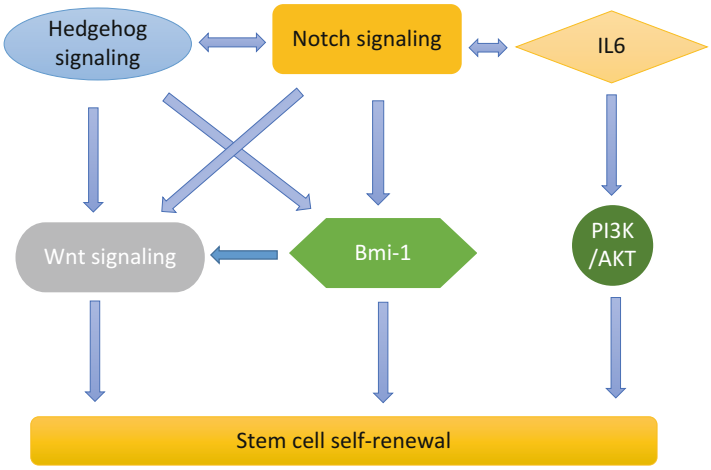

Fig. 6.1 Interaction between the signaling pathways that regulate stem cell self-renewal

5 Conclusions and Future Perspectives

In this chapter, we demonstrated that several transduction pathways, including Hedgehog, Notch, Wnt, the transcription factor Bmi-1, PI3K/Akt, and IL6 regulate self-renewal pathways in normal stem cells, while in CSCs these pathways are normally dysregulated due to accumulated mutations and epigenetic changes. Conventional cancer therapies normally target aberrant pathways in the rapid proliferating bulk tumor cells, but often spare the CSCs leading to tumor recurrence and metastasis. Therefore, the design of new therapies must be based on targeting the signaling pathways that affect both CSCs as well as bulk tumor cells. One challenge here is understanding the signaling pathways through which CSCs regulate their self-renewal and maintenance and hence tumor growth and metastasis, so we can use that as a target to abrogate CSCs. Another challenge is the combination of drugs targeting different pathways for better treatment outcome. The combination of conventional cancer therapies together with specific CSC targeted therapies brings the promise of eradicating a cancer with no possibility of recurrence.

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Part II
Cancer Stem Cells in Human Malignancies

Chapter 7
Breast Cancer Stem Cells

Giorgia G. Silveira, Joao Paulo Oliveira-Costa, and Alfredo Ribeiro-Silva

Abstract Breast cancer is the second most common cancer worldwide and the most frequent among women, and despite several efforts, continues to show a poor prognosis and high mortality. Breast cancer stem cells are a small group of cells among the bulk of the tumor that shows stem cell properties, like self-renewal and immortality. They are believed to be involved in cancer promotion, development, and have been studied as important molecules either for diagnosis or treatment. Although several markers were studied to identify breast cancer stem cells, it stills unclear if the common markers CD44, CD24 and ALDH1 could be used to determine these cells. We will cover in this chapter the possible mechanisms involved in breast cancer stem cells theory, its markers and their potential as prognostic or predictive molecules, in terms of survival and treatment of breast cancer.

Keywords Breast cancer • Cancer stem cells • CD44 • ALDH1 • Steroid hormone receptor • Therapy resistance

1 Breast Cancer Biology and Pathology

The human mammary gland is a specialized organ formed by ducts that branch out from the nipple, and can be split into terminal duct lobular unit, which is considered the breast anatomo-functional unit, and major ducts. Unlike other organs, the breast is not fully developed at birth and the branching of the ductal system occurs in female puberty. During the menstrual cycle, breast undergoes intense hormonal influence and the epithelium responds by regional proliferation, differentiation, and apoptosis (Andres and Strange 1999). The ducts and lobules of mammary gland adults are formed by two types of cells, luminal epithelial and myoepithelial and two types of stroma, the interlobular formed by fibrous connective tissue and

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adipose tissue and intralobular, which consists of fibroblast-like cells and lymphocytes (Böcker et al. 2002). These specialized epithelium and stroma may give rise to benign and malignant lesions in breast.

Breast cancer is the second most common cancer worldwide and the most frequent among women. In 2008, the estimated incidence of breast cancer worldwide was 1.38 million cases and the number of deaths was 458,400 cases (Jemal et al. 2011). In the United States the estimate for 2014 is 235,030 new cases and 40,430 deaths (Siegel et al. 2014). Breast cancer is a heterogeneous disease that presents different subtypes and distinct clinical outcomes. Adenocarcinomas represent greater than 95 % of all breast malignances and can be classified as invasive ductal breast carcinoma and ductal carcinoma *in situ* (DCIS). DCIS is characterized by a group of tumor cells that it is limited by the basement membrane to the mammary ducts and lobules (Fig. 7.1). Morphologically, DCIS can be divided accordantly five architectural subtypes: noncomedo DCIS (papillary, micropapillary, cribriform, solid) and comedocarcinoma. The presence of pleomorphic cells with hyperchromatic nuclei and areas of central necrosis characterizes comedocarcinoma and chronic inflammation and periductal concentric fibrosis are common in this type of carcinoma. In cribriform DCIS, intraepithelial spaces presents regular form and are evenly distributed while solid DCIS completely fills the involved spaces. The primary characteristics of micropapillary DCIS is the bulbous protrusions without a

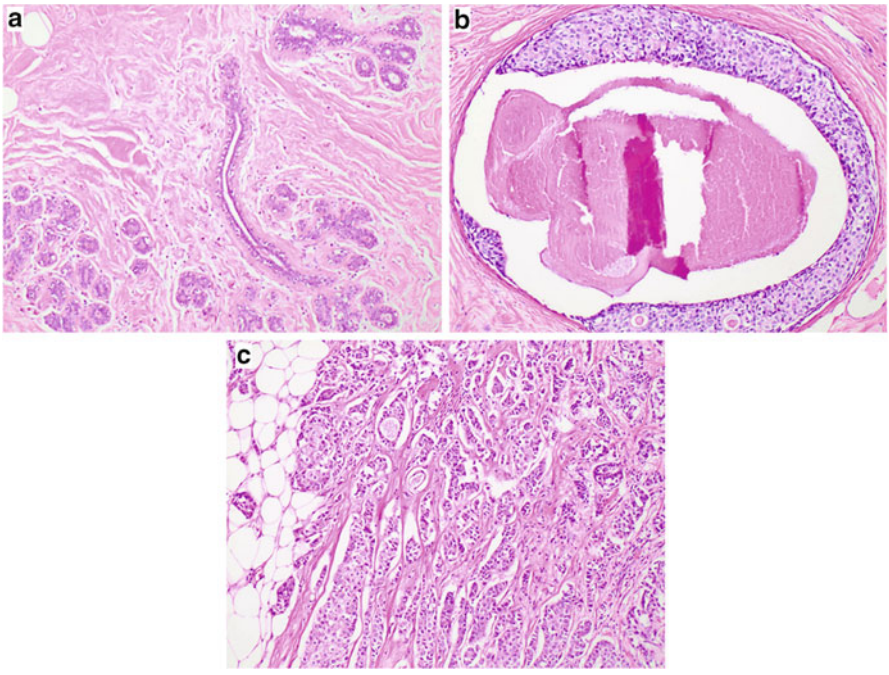

Fig. 7.1 Hematoxilin-eosin staining (**a**) normal breast, (**b**) ductal carcinoma in situ, (**c**) invasive ductal carcinoma

fibrovascular core and papillary DCIS grows along fibrovascular cores that typically lack the normal myoephitelial cell layer (Kumar et al. 2010).

Invasive carcinoma is a group of breast carcinomas that infiltrates the stroma, regardless of the coexistence of *in situ* component. Invasive ductal carcinoma is the most common type of breast cancer representing over 80 % of all tumors. These tumors are very heterogeneous and still present only 60 % of five years survival. In an effort to classify these tumors in order to guide treatment, Perou et al. (2000) demonstrated by cDNA microarray technique, that breast tumors may be grouped according to their gene expression in ER^+/luminal-like, basal-like, $Erb\text{-}B2^+$ and normal breast.

The profile ER^+/luminal-like is a group of tumors positive for hormone receptors and negative for HER2. $Erb\text{-}B2^+$ profile is negative for hormone receptors and positive for HER2 and basal-like is a group of breast malignant lesions that are negative for hormone receptors and HER2. Normal breast is a group of tumors that do not qualify for these subtypes (Brasileiro-Filho 2006). Recent studies performed by different groups demonstrate that each subtype presents different risk factors. In addition, these profiles show correlation with response to treatment, prognosis and patient clinical outcome (Hu et al. 2006; Polyak and Hahn 2006; Sørlie et al. 2006).

2 Initiation and Disease Progression

The most accepted theory to explain the development of cancer says that malignant tumors are the result of sequential mutations that may arise from genetic instability and/or environmental factors (Reya et al. 2001), however the exact mechanism of tumor formation is still unknown. Understand the tumor initiation and progression is fundamental to achieve better treatment options. In breast tumors, the stratification proposed by Perou et al. (2000) impacted dramatically on treatments and outcomes, since it is now possible to treat patients based on their subtype of cancer. However, target treatment and chemotherapy still remain highly unpredictable. As previously mentioned, breast tumor are a very heterogeneous disease with different subtypes that are characterized by different morphology, molecular profile, and expression of specific markers. Currently two models are studied in an attempt to explain the intertumor heterogeneity, the genetic mutation model and the cell of origin model (Visvader 2011).

The genetic mutation model is based on the premise of accumulation of different genetic and epigenetic mutations in the same cell, giving rise to different tumor phenotypes. The cell of origin is a normal cell that undergoes the first mutation that may lead to cancer. Thus, in cell of origin model, different cells of the same tissue undergo an initial mutation and give rise to different tumor subtypes. It is crucial to note that cancer stem cells (CSCs) are a cellular subset in the bulk of tumor that uniquely sustains malignant growth, i.e., they are cells responsible for tumor propagation, not tumor initiation (Visvader 2011). Self-renewal and immortality characteristic of cancer stem cells are fundamental in the maintenance and

propagation of the tumor. In several solid tumors and leukemia, the hierarchical organization of cancer cells is widely accepted and it is believed that these tumors are maintained by a subpopulation of self-renewing cells that can generate all of tumor cells, the CSCs.

Recent studies show that either stem or progenitor cells can be a target for first mutation in solid tumors. Lineage tracing studies demonstrated in pancreatic ductal adenocarcinomas and prostate, intestinal and basal cell carcinomas probable cells of origin. It is important to observe that in most of these studies the cell of origin in which transformation occurs remains unknown although these studies have identified the lineage in which the cancer originates (Barker et al. 2009; Gidekel Friedlander et al. 2009; Mulholland et al. 2009; Molyneux et al. 2010).

3 Identification of Breast Cancer Stem Cells

As far as the current knowledge in cancer stem cells field goes, a single cell with a specific profile of cell-surface markers can repopulate rodent's mammary glands (Shackleton et al. 2006; Stingl et al. 2006). Therefore, such cell-surface markers could be useful in the characterization of cells regarding its stemness, and also providing further insights into different signaling pathways involved in cancer stem cells biology. Some human cells have been shown to be able to repopulate mammary fat pads in NOD/SCID mice, and these cells showed a CD24high/CD49fhigh/DNERhigh (Delta and Notch-like epidermal-growth-factor related receptor) profile, while a lower DNER seems to avoid these cells of repopulating fat pads (Pece et al. 2010). Also, these CD24high/CD49fhigh/DNERhigh, when positive to DLL1, were shown to develop tumors in NOD/SCID mice and interestingly were enriched in high-grade tumors. It stills not clear whether these cell-surface markers are functionally altered in breast cancer, or if they are only phenotypical markers of BCSCs. Although some markers could be considered only phenotypical markers, others could also be involved in breast cancer stem cells biology, such as CD44, CD133 and aldehyde dehydrogenase 1 (ALDH1). The principal markers and pathways related to stem cell characterization are summarized in Table 7.1.

CD44 glycoprotein is a cell surface receptor for hyaluronic acid, and is believed to be involved in cell adhesion, migration, and metastasis in certain types of tumors (Shipitsin et al. 2007). CD44 cell-surface marker has been used to identify putative CSCs in breast tumors (Shipitsin et al. 2007), similarly to what was found in other tumor types, such as prostate (Collins et al. 2007), pancreatic (Li et al. 2007), and head and neck squamous cell carcinomas (Prince et al. 2007). Shipitsin et al. (2007) found that $CD44^+$ tumoral mammary cells were associated with more invasive, proliferative, and angiogenic status, predicting an aggressive tumoral cell behavior. Moreover, there was a correlation between $CD44^+$ tumoral cells and decreased patient survival.

CD24 is a mucin-like adhesion molecule usually expressed by neutrophils, pre B-lymphocytes and a large variety of solid tumors. The molecular mechanisms

Table 7.1 Cell surface markers and signaling pathways in cancer stem cells (Morrison et al. 2008)

Factor	Characteristics
CD44	CD44 is involved in cellular adhesion, motility, and metastases
EpCAM/ESA	Epithelial cell adhesion molecule/epithelial surface antigen is expressed on mammary tissue and tumors
CD49f (α6-integrin)	Involved in basal and endothelial cell distribution and is a candidate stem cell marker
CD133 (prominin-1)	Cell surface glycoprotein with an unknown function in cancer stem cells and its expression is documented for various types of cancer
ALDH1	Aldehyde dehydrogenase-1 plays a role in the differentiation of stem cells and its activity predicts poorer clinical outcomes
CXCR4	Chemokine receptor involved in metastasis and its expression is increased in mammospheres
ER	Expressed on breast cancer cells, mammary progenitors, and breast cancer stem cells
Delta/notch pathway	Involved in cell fate development and is expressed in stem cells and early progenitor cells
Wnt signaling pathway	Participates in stem cell self-renewal and its overexpression can lead to epithelial and mammary tumors
Hedgehog/patched pathway	This pathway is involved in embryonic growth and cell fate determination

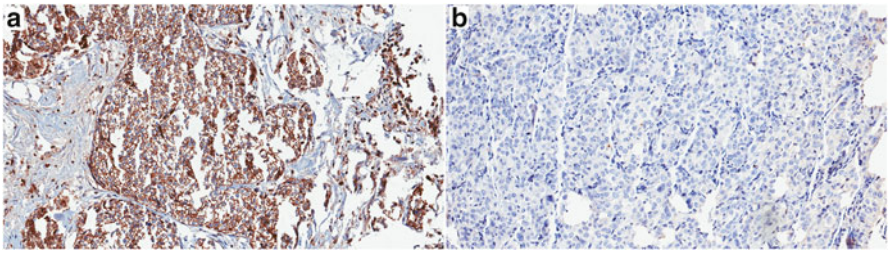

Fig. 7.2 Immunohistochemistry showing (**a**) membranous staining for CD44, (**b**) absence of staining for CD24 in invasive ductal carcinoma

underlying CD24 participation in carcinogenesis still not fully understood, but it is believed that CD24 enhances the metastatic potential of malignant cells, partially because it has been identified as a ligand of P-selectin, an adhesion receptor present on activated endothelial cells (Lim and Oh 2005). It was demonstrated that intracytoplasmic expression of CD24 was highly associated with colonic adenocarcinoma, gallbladder, and ovary compared to the adenoma found on those organs, and also with positive nodal status compared to negative nodal status of the colonic adenocarcinoma (Lim and Oh 2005). Positive or negative CD24 expression has been used together with other markers to identify CSCs in tumors, and some studies defined the phenotype of pancreatic CSCs as $CD24^+/CD44^+$ (Li et al. 2007; Zou 2008) while in breast and prostate cancer, putative CSCs were found with a $CD24^-/CD44^+$ phenotype (Al-Hajj et al. 2003; Hurt et al. 2008) (Fig. 7.2).

CD133, also called prominin 1 (PROM1), was discovered as a marker of hematopoietic stem cells and was later used to select putative CSCs in several tumor types (Singh et al. 2004; Mizrak et al. 2008). In brain tumor, *in vitro* $CD133^+$ cells could successfully grow under unattached conditions, with neurosphere-like formations, whereas CD133- cells could not. *In vivo*, the $CD133^+$ cell fraction isolated from medulloblastomas and glioblastomas and injected into the brains of NOD/SCID mice, could maintain its capability of initiating tumors, with phenotypic similarity between the new and original tumors (Singh et al. 2004). Besides its role in brain tumors, other studies show that CD133 may play a role in migration and asymmetric division of stem cells (Balic et al. 2006; Beckmann et al. 2007).

A hypothetical model of tumor progression to metastatic disease in breast cancer considers that metastasis can be initiated by invasive $CD44^+$ breast cancer cells and that tumors rich in $CD44^+$ cells have a significantly worse clinical outcome (Shipitsin et al. 2007; Shipitsin and Polyak 2008). In agreement with this assessment, it has been hypothesized that breast cancers of basal-like phenotype, which carry a poor outcome and great resistance to current therapy, are enriched with $CD44^+$ cells (Sørlie et al. 2001; Shipitsin et al. 2007). In the last years, specific populations of breast cancer cells with stem cell-like features and tumorigenic characteristics were identified and investigated in some *in vivo* models. Al-Hajj et al. (2003) were the first to isolate the CSCs in solid tumor and identified human tumorigenic breast CSCs with an enriched $CD44^+/CD24^-$/low/ESA^+ phenotype. As few as 200 of these cells were capable of forming new tumors when implanted in the mammary fat pad of female NOD/SCID mice. Conversely, 20,000 cells isolated from the same tumor that did not display this phenotype were unable to form tumors. Also, the $CD44^+/CD24^-$/low/ESA^+ cells isolated from the xenograft tumors were able to be transferred to secondary and subsequent hosts forming new tumors with similar phenotypes, demonstrating the capacity for maintenance of self – renewal and tumorigenic properties (Al-Hajj et al. 2003). Consistent with these results, Dontu et al. (2003) and Ponti et al. (2005) found that isolated $CD44^+/CD24^-$ human breast cancer cells can also form tumor mammospheres and maintained the capability of propagation *in vitro*. Furthermore, clinical studies indicate that $CD44^+/CD24^-$ tumor-initiating cells express an invasive gene signature and may be associated with distant metastases (Abraham et al. 2005; Balic et al. 2006; Liu et al. 2007). Although Honeth and co-workers have shown a relationship between $CD44^+$/CD24- immunophenotype and basal-like tumors, not all basal-like tumors contain $CD44^+$/CD24- cells, which sheds light in the discussion between whether only a phenotype can be representative of all different clones that arise with tumor development (Honeth et al. 2008).

ALDH1 is a detoxifying enzyme responsible for intracellular oxidation of aldehydes. According to Sophos and Vasiliou (2003), ALDH1 may have a role in early differentiation of stem cells through its function in oxidizing retinol to retinoic acid. Retinoic acid signaling pathway is linked to cellular differentiation during development and plays a role in stem cell self-protection (Croker et al. 2009). ALDH1 activity can provide a common marker for both normal and malignant stem cells. Cells with high ALDH1 activity have been associated with several types of human hematopoietic and neural stem cells (Armstrong et al. 2004; Corti et al. 2006).

Confirming these findings, Nagano et al. (2007) demonstrated that the ALDH1 enzyme was able to identify rapidly dividing cells representing a progenitor cell population in human umbilical cord blood and bone marrow. Therefore, in agreement with Croker et al. (2009), the use of ALDH1 activity detection as a purification strategy allows an efficient isolation of normal and malignant human stem cells based on a developmentally conserved stem cell function.

In the mammary gland, Ginestier et al. (2007) demonstrated that ALDH1 is a marker of stem/progenitor cells of the normal human breast and breast carcinomas. More recently, the activity of cytosolic ALDH1 has also been shown to be a reliable marker of CSCs in several types of solid tumors, including tumors of the breast, colon, liver, head and neck, prostate and the bladder regions, and at least in some of these tumors, high ALDH1 activity is related to a poor prognosis (Burger et al. 2009; Ginestier et al. 2007; Huang et al. 2009; Lingala et al. 2010; Lugli et al. 2010; Clay et al. 2010; Deng et al. 2010; Li et al. 2010; Su et al. 2010). ALDH1-positive tumors showed a lower overall survival when compared to ALDH1-negative tumors, in a cohort of 577 breast cancer patients (Ginestier et al. 2007). Some studies has shown a partial overlap between the $CD44^+/CD24^-/lin^-$ and $ALDH1^+$ populations, with cells expressing $CD44^+/CD24^-/lin^-/ALDH1^+$ phenotype being able to form tumors starting from as few as 20 cells. Furthermore, the ALDH1-positive population isolated from human breast tumors demonstrated the ability to generate tumors in NOD/SCID mice.

Expression of $CD44^+/CD24^-$, $ALDH1^+$, CD49fhigh/DLL1high/DNERhigh, have all been associated with stem cell activity in breast cancer. Differences in evolutionary pathways, and limitations with stem-cell assays, might lead to misidentification of different subpopulations of cells as CSCs. In a first step to avoid misclassification, it is important to avoid automatically considering all of these markers as "stem cell markers". Just as an example, the immunophenotype $CD44^+CD24^-$ could be acquired by cells that have undergone epithelial to mesenchymal transition, not only by clonal evolution of CSCs. Thus, it is important to further validate BCSC markers in order to avoid misclassifying as CTCs what could be just an aggressive variant of a different cancer cell with properties of CSCs.

4 Breast Cancer Metastatic Pathways

After characterization of cancer stem cells, several studies reveal that these cells may be responsible for metastasis and recurrent relapses after radiotherapy and chemotherapy (Liu et al. 2010; Sansone et al. 2007; Wicha et al. 2006). It is believed that tumor microenvironment plays a pivotal role in regulation of either chemotherapy and radiotherapy resistance following treatment, presumably due to inflammatory cytokines that stimulate CSCs self-renewal, thus promoting tumor metastasis (Sansone et al. 2007; Ginestier et al. 2010). Among the large amounts of factors produced by different cells in the tumor microenvironment, Wnt is known to be involved in CSCs self-renewal, and also plays an important role in CSCs initiation and maintenance

(Jamieson et al. 2004; Ginestier et al. 2007; Abrahamsson et al. 2009). Hedgehog (Hh) signaling pathway was also shown to be active in pancreatic mouse xenograft, and regulate the maintenance of human leukemic stem cells. The knockdown of Hh defector smoothened (Smo) caused the elimination of stem cells in chronic myeloid leukemia (Zhao et al. 2009). Therefore, besides the regular phenotypes presented by potential CSCs, it is believed that part of these cells may have a different phenotype, or even have disturbance in another pathways leading to the ability of metastasizing. Targeting these metastasizing cells is an important field of application for BCSCs, as metastasis is a leading cause of mortality in breast cancer patients.

Circulating tumor cells (CTCs) can very often be detected in patients with metastatic disease, and the presence and number of CTCs in blood are associated with worse survival and prognosis (Cristofanilli et al. 2005; Jatana et al. 2010). Given that metastasis is known to be an organ-specific process that is developed by certain types of cells, in the breast it has been shown that the same pathways involved in hematopoietic cells migration could be involved in metastatic breast cancer. More specifically, CXCR4, a chemokine receptor that binds to CXCL12, was expressed in both metastatic breast cancer and neuroblastoma, and also increased in mammospheres (Müller et al. 2001; Dontu et al. 2003; Geminder et al. 2001). Interestingly, the most common metastasis organs in breast cancer patients showed the higher expression levels of the CXCR4 ligand CXCL12 (Müller et al. 2001). Therefore, CXCR4/CXCL12 pathway involvement in breast cancer metastasis highlights the "seed" and "soil" theory of specific metastasis to determined organs, and could represent important target to future therapies.

Although a subpopulation of CTCs are hypothesized to initiate metastatic carcinoma, and the presence/number of CTCs in patient's blood is an indicator of poor prognosis, the different phenotypes associated with these CTCs and their importance in metastasis are only now beginning to be elucidated (Lustberg et al. 2014); Theodoropoulos et al. (2010) showed that 35 % of CTCs in 20 patients out of 30 expressed the BCSC $CD44^{+}CD24$ low phenotype, and 17.7 % of patients showed an ALDH1high/CD24low phenotype. Phenotypes associated with breast CTCs have been shown to be as important as enumeration. Lustberg et al. (2014) showed that patients presenting CTCs that were negative to CD45 (lymphocyte common antigen), positive to cytokeratins (presumable of epithelial origin) and negative to EpCAM had a worse overall survival. Also, several patients had no EpCAM positive cells, while presenting $CD45\text{-}CK^{+}$ cells (Lustberg et al. 2014). As the only FDA-approved CTC separation technology is based on EpCAM positivity, further studies will be helpful to determine whether breast cancer CTCs might have epithelial CTCs and other CTCs that have undergone epithelial-mesenchymal transition (EMT).

EMT and its reverse process, called mesenchymal-epithelial transition (MET), play a central role in embryonic development, where the mesoderm generated by EMT develops into multiple tissue types, and through MET, mesenchymal cells generate epithelial organs (Davies 1996). In recent years, embryonic transcription factors were involved in several malignant processes such as motility, invasiveness, and resistance to apoptosis (Cheng et al. 2007; Comijn et al. 2001; Savagner et al.

2005). EMT can be induced through several factors, like TGF-β or receptor tyrosine kinase ligands that trigger changes in downstream target networks, like the up regulation of proteins Snail, Slug, and Twist (Peinado et al. 2007). These proteins bind to the promoter of E-cadherin gene, where they facilitate chromatin condensation and subsequent transcriptional repression of E-caderin. Therefore, reduced expression of E-cadherin causes the breakdown in adherents junctions, together with loss of cell polarity, and a b-catenin mediated change in gene expression that will result in the expression of classical markers of mesenchymal cells, such as fibronectin and vimentin (Zeisberg and Neilson 2009; Singh and Settleman 2010).

There is evidence that EMT could generate cells with properties of CSCs, including an 30-fold increase in the ability of mammosphere formation, formation of soft agar colonies and a more efficient formation of tumors in mice (Radisky and LaBarge 2008). Also, transformed mammary human epithelial cells that have forced to undergone EMT showed similar expression of EMT markers compared to stem-cell like cells isolated from human mammary epithelial cells (Radisky and LaBarge 2008). In fact, it is known that some pathophysiological conditions can trigger differentiated cells to acquire a stem cell-like phenotype, presumably through EMT induction. As stated before in this chapter, several signaling pathways involved in metastatic processes are involved in EMT signaling, such as Wnt, Notch and Hh (Fig. 7.3), responsible for the control of normal and CSC maintenance and renewal (Huber et al. 2005; Peacock and Watkins 2008; Singh and Settleman 2010). In

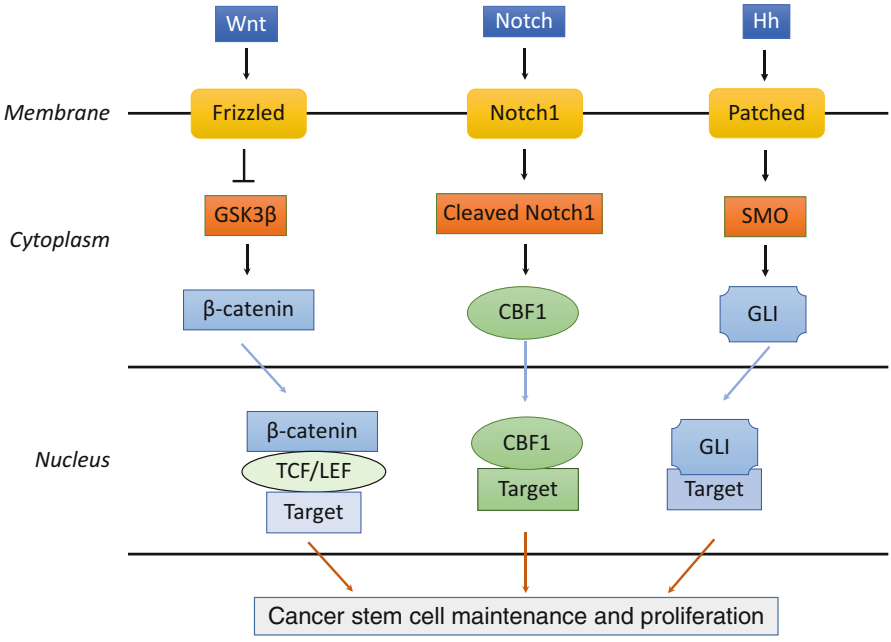

Fig. 7.3 Pathways involved in cancer stem cells maintenance and proliferation (Adapted from Geng et al. 2014)

breast cancer, cells collected from pleural effusions of patients were enriched for a $CD44^+/CD24$ low CSC-like population (Al-Hajj et al. 2003). Corroborating the idea of an EMT-associated pathway controlling CSC maintenance, CD44 is a known target gene for beta-catenin/TCF-4 (Wielenga et al. 1999).

5 Steroid Hormone Receptor Expression of Breast Cancer Stem Cells

As stated early, ovarian hormones such as estrogen and progesterone have a profound influence in breast cancer risk, and its influence is shown as several patients benefited for decades from endocrine therapies. The cellular mechanisms associated with these observations are not well understood to date, but a few studies have recently addressed how BCSCs could be involved in hormonal signaling/Asselin-Labat and collaborators have shed light on whether BCSCs are modulated by hormonal stimuli, in mouse. Mouse mammary stem cells showed to be highly responsive to hormonal stimuli although not expressing estrogen or progesterone receptors (Asselin-Labat et al. 2006).

The identification of cell surface markers for BCSCs as well as the development of cell lines and mouse models has facilitated how we understand BCSCs relationship with hormonal therapy. *In vitro* studies have used established cell lines and primary tumors and suggested that SCs are relatively resistant to both radiation therapy and chemotherapy (Dave and Chang 2009). Considering the expression of stem cell surface markers before and after neoadjuvant chemotherapy, the fraction of $CD44^+CD24^-$ cells and mammosphere-forming cells are higher after treatment (Li et al. 2008), and expression profile of primary tumor after therapy resembles that seen before treatment in either $CD44^+CD24^-$ cells and mammosphere-forming cells (Creighton et al. 2009). This gene expression signature is interestingly also found in tumors remaining after neoadjuvant endocrine therapy utilizing aromatase inhibitors (Creighton et al. 2009). These findings are supportive of preclinical findings suggesting that some ER-expressing breast cancer cell lines could contain ER-negative CSCs (Horwitz et al. 2008). This expression pattern is similar to the one found in normal human breast where ER-negative stem cells lead to ER-positive luminal cells (Asselin-Labat et al. 2006), supporting the idea that estrogen may indirectly trigger the production of paracrine factors by ER-positive tumor cells, affecting thus ER-negative BCSCs.

This hypothesis was recently corroborated by Harrison and co-workers, who have found similar results, although showing an increase in BCSCs in presence of oestrogen (Harrison et al. 2013). First, the authors found that BCSCs are usually negative or showed low expression of ER, although the cells were also susceptible to oestrogen treatment. The authors also proposed that the paracrine effects of oestrogen stimuli could be at least in part given by Notch and EGF signaling networks. The interplay between Notch and EGFR signaling was demonstrated as an additive effect in human ductal carcinoma *in situ*, demonstrating that Notch and EGFR

(ErbB1/2) both play a role in ductal carcinoma *in situ* stem cell activity, and also that cross talk between the two pathways in DCIS occurs regardless of ErbB2 receptor status and inhibition of Notch and ErbB1/2 was more efficacious than either alone (Farnie et al. 2013).

Important experiments have also demonstrated a role for the breast cancer susceptibility gene BRCA1 in the differentiation of ER- stem cells into ER^+ luminal epithelial cells (Liu et al. 2008). Deletion of BRCA1 showed an impact on the transition of ER- stem cells into ER^+ progenitor cells. Furthermore, it has been shown that heterozygous mutations in the BRCA1 gene, besides predisposing women to breast and ovarian cancer, could lead to tumors often lacking expression of hormonal receptors (ER, PgR) and HER-2 (Narod and Foulkes 2004). This suggests a model in which modifications in BRCA1 could lead to an increase in ER- stem cells, leading to a more basal phenotype. However, greater than two thirds of breast cancer tumors are ER^+ and the majority of these tumors are dependent on oestrogen for growth and thus can be treated with hormonal therapy (Buzdar et al. 2004).

Regarding HER-2 (ErbB2) expression in BCSCs, Duru et al. (2012) showed important results when comparing MCF-7 derived $HER2^+/CD44^+/CD24^-$/low cells with HER- BCSCs. The $HER2^+/CD44^+/CD24^-$/low cells showed elevated ALDH activity and aggressiveness, tumor sphere formation and *in vivo* tumorigenesis. The aggressive phenotype and radioresistance of $HER2^+/CD44^+/CD24^-$/low cells were markedly reduced by inhibition of HER2 or Herceptin treatments. More important, clinical breast cancer specimens revealed that cells co- expressing HER2 and CD44 were more frequently detected in recurrent (84.6 %) than primary tumors (57.1 %), showing that HER2-mediated pro-survival signaling network could responsible for an aggressive phenotype of breast cancer stem cells (Duru et al. 2012). In vitro studies also showed that HER2 inhibition with trastuzumab antibody reduced breast cancer stem cells ($CD44^+/CD24^-$ cells) from MCF7 cultures. The role of HER2 in the regulation of CSCs in luminal tumors was showed using mouse tumor xenografts. HER2 expressing MCF7 cells had significantly greater tumor initiating capacity than HER2 non-expressing cells. The effects of trastuzumab were highly dependent on administration time, with better results in early treatment. These results emphasize the pivotal role of CSCs in mediating tumor recurrence following adjuvant therapy, an important prediction of the CSC hypothesis (Ithimakin et al. 2013). These results corroborate Korkaya et al. (2008) that had also shown that HER2 overexpression in normal mammary epithelial cells could increase the proportion of stem/progenitor cells, through formation of mammospheres and expression of ALDH as well as the formation of hyperplastic lesions in humanized fat pads of NOD/SCID mice. The effects of HER2 overexpression on breast cancer stem cells are blocked by trastuzumab in sensitive, but not resistant, cell lines, an effect mediated by the PI3-kinase Akt pathway (Korkaya et al. 2008).

These models suggest that the diversity seen in tumor types among different patients could be a result of transformation events occurring in different lineages of breast cancer stem cells, and further determining hormonal receptor status of BCSCs could have important implications on the treatment of disease, especially those resistant to chemotherapy.

6 Breast Tumor-Associated Antigens

Immunotherapy is expected to stimulate the immune system to recognize and eliminate tumor cells, and has been focus of research for several years but only recently has gained interest. Many vaccines targeting solid tumors have been employed with varying success both pre-clinically and clinically in the treatment of cancer (Mocellin et al. 2004). The increasing understanding on how the immune system is related with cancer, and the identification of tumor-associated antigens (TAAs), that may be used as targets for therapy led to an increased interest in the discovery of further targets for vaccines (Morrison et al. 2008). In breast cancer, there are evidences that the immune system is involved in cancer surveillance, but it is impaired by tumor-produced factors, in order to avoid this immunosurveillance (Zitvogel et al. 2006). In this context, the role of dendritic cells (DCs) is central due to their role in innate immunity, generating both humoral and cellular responses. Docs are antigen-presenting cells that present antigens/epitopes to T-cells. Depending of the antigen that is presented, the cytolytic T-cell can drive the effective immune response the cell expressing the antigen.

Breast cancer TAAs studied to date includes antigens such as carcinoembryonic antigen, NYBR-1, HER-2, MUC-1, telomerase, survivin and p53. Exactly the same way it happens in CTCs, serological identification of antigens that can be explored as vaccine targets is dependent of the different types of antigens expressed by different subtypes of cells. This means that even in a single patient, odds are that a single antigen will not be able to be representative of all different cells, and several cells not presenting the given antigen will evade T-cell identification and immune responses (Morrison et al. 2008). Targeting the CSC pool of cells could, at least in theory, eliminate this population.

Although preclinical data has shown that DCs induce effective antitumor responses, clinical trials overall have been disappointing, with a lack of objective tumor response reported in at least 12 of 35 trials (Mocellin et al. 2004). One trial with breast and ovarian cancer patients using TNFa-matured, monocyte-derived DCs pulsed with MUC1 or HER-2/neu raised immunological responses in patients with advanced diseases that were pretreated by multiple cycles of chemotherapy, including high-dose chemotherapy and autologous stem cell transplantation, indicating that peptide-pulsed DC vaccinations could also be successfully applied after intensive or even high-dose chemotherapy to eliminate residual disease (Brossart et al. 2000). Another antigen-defined approach makes use of transfer of antitumor T-cells cultured ex vivo and identified to be active against target antigens. Recently, the examination of adoptive transfer of HER-2-specific T-cell clones clinically suggests the potential to use an antigen-specific therapy to eliminate specific single tumor cells but that additional treatments are needed to reduce the solid tumor (Bernhard et al. 2008).

To avoid these problems, it is believed that targeting multiple antigens is going to be required. From what is known regarding clinical trials, it appears that several epitopes need to be targeted simultaneously for an effective therapy through the use

of a polyvalent vaccine that could target more antigens simultaneously. One way to achieve this is to make use of whole tumors in the vaccine, as used by O'Rourke and colleagues (2007). In patients with stage IV melanoma using a DC/irradiated tumor vaccine has demonstrated a complete remission of disease in 3 out of 46 patients and a partial remission in other 3 patients (O'Rourke et al. 2007).

Mammospheres have shown the expression of markers, such as CD44, CD49f, and ALDH1 among many others that could potentially be used to target BCSCs (Dontu et al. 2003). Interestingly, Wright et al. (2008) showed that BRCA1-deficient murine breast tumors contain heterogeneous cancer stem cell populations. Some tumors contained cells with a $CD44^+$/CD24 low phenotype and also different cells with $CD133^+$ phenotype. Both $CD44^+$/CD24 low and $CD133^+$ phenotypes rapidly formed tumors in nonobese diabetic/severe combined immunodeficient mice, whereas 50- to 100-fold higher numbers of parental or stem cell depleted cells were required to form few, slow-growing tumors. Importantly, both populations of cells expressed the stem cell-associated genes Oct4, Notch1, Aldh1, Fgfr1, and Sox1. Although this study shows a variety of markers shared by different cells, BCSCs heterogeneity still a factor that might have to be take into account when developing strategies to increase immunosurveillance.

7 Breast Cancer Stem Cells and Therapy Resistance

From a clinical standpoint, cytotoxic chemotherapy can potentially kill all tumor cells if it not had its potential reduced by crucial factors, such as reduced systemic availability, problems with drug delivery and other resistance mechanisms (Pinto et al. 2013). Intrinsic tumoral resistance is that where tumors are either insensible to a given signaling pathway being target of the drug, or even its ability to retain the drug for enough time to exert its effects. An example of intrinsic resistance is the resistance of triple negative breast tumors to conventional therapy. Acquired resistance to therapy is that where tumors are initially responsive to regular therapy, but this response are not sustainable for long periods. This means that these patients will have a favorable response to initial therapy, but will soon see their tumors relapse, as they become resistant to the first line of treatment (Fulda 2009; Coley 2008; Saunders et al. 2012). In either case, those patients are associated with a poor survival rate, and several efforts are being made to overcome resistance and elevate their prognosis. Recently, several articles suggested that epithelial to mesenchymal plasticity may be one of the mechanisms involved in chemotherapy evasion by cancer cells. After hormonal or chemo therapy, the subset of remaining cells was shown to be characterized by expression of either epithelial (cytokeratins) and mesenchymal markers (such as vimentin), which is in accord with the finding that chemotherapy and hormonal therapy have little to no effect in cells that have undergone EMT (Charafe-Jauffret et al. 2009; Hollier et al. 2009). This plasticity between epithelial and mesenchymal states may be indeed an important mechanism by which tumor cells could evade chemo and/or hormonal therapy. In this scenario,

undifferentiated cells that poorly responds to chemotherapy could use transient mechanisms (such as epithelial-mesenchymal plasticity) to avoid been affected by treatment, thus originating recurrences and relapses formed by subpopulations associated with poorly prognosis (Pinto et al. 2013). Supporting this proposed mechanism, overexpression of plasticity factors such as Snail and Twist in breast cancer cell lines was able to turn this cell insensible to treatment with paclitaxel and doxorubicin (Li et al. 2009; Cheng et al. 2007). Conversely, restoration of e-cadherin expression in mesenchymal MDA-MB-231 breast cancer cell line turns the cells sensible to doxorubicin treatment (Tryndyak et al. 2010).

Diessner et al. (2014) have recently shown that treatment of $CD44^{+}CD24^{-}$HER2 low BCSCs with antibody-drug conjugate T-DM1 (trastuzumab conjugated to mertansine) led to a high sensitivity of these cells to T-DM1. The authors also found that preexisting $CD44^{+}$CD24 low cancer stem cells were depleted by concentrations of T-DM1 that did not affect the other cell types from the bulk of tumor and that colony formation was efficiently suppressed. Also, when tumor cells were co-cultured with natural killer cells, the antibody-dependent cell-mediated cytotoxicity was enhanced, and EMT-mediated induction of stem cell-like properties was prevented in differentiated tumor cells.

$CD44^{+}CD24^{-}$ stem cells showed a relative resistance to ionizing radiation, and side populations are known to be more resistant than non-side populations (Phillips et al. 2006; Woodward et al. 2007; Diehn et al. 2009). The enrichment of BCSCs has been described as contributing to cisplatin resistance and tumor progression in BRCA1/p53 model and also as being caused by common neoadjuvant chemotherapy, while neoadjuvant treatment with lapatinib may decrease BCSC enrichment in HER-2 positive tumors (Yu et al. 2007; Shafee et al. 2008; Li et al. 2008). These findings suggested that conventional therapy could be an important factor in selecting resistant clones of BCSCs that may favor future disease recurrence and progression (Al-Ejeh et al. 2011). The precise mechanisms behind the concept that BCSCs may be responsible for at least some breast cancer recurrences are still unknown, but several mechanisms have been implicated.

Besides EMT and radiation resistance, other mechanisms were associated with treatment resistance, such as ABC transporters expression abnormalities, and key pathways possible involved in this resistance. ATP-binding cassette (ABC) transporters are membrane pumps that can transport small molecules such as dyes or cytotoxic drugs out of the cell (Chen et al. 2013), and they are comprised of important proteins such as multidrug resistance proteins (MRP), breast cancer resistance protein (BCRP/ABCG2) and P-glycoprotein (P-gp/ABCB1) (Deeley et al. 2006). These transporters can be identified by treating cells with Hoechst 33342 dye, and observing cells that overexpress ABC transporters to expulse the dye. This fraction of cells is known as side population (SP) and these SPs have a capacity of tumorigenesis greater than non-SP cells (Chen et al. 2013). ABC transporters are usually associated with multidrug resistance (MDR) due to its ability to transport the cancer therapies out of the cell, therefore reducing its concentration inside the cancer cell, and the effects of therapy (Matsui et al. 2008; Kruger et al. 2006; Cho et al. 2008).

Researchers have designed several different methods to target MDR, in order to overcome drug resistance, such as verapamil. Verapamil is an L-type calcium channel blocker of the phenylalkylamine class, commonly used to treat hypertension. Promising therapeutic effects has been achieved in tests with verapamil associated with anti-tumor drugs, as doxorubicin and paclitaxel.

Investigators have identified molecules such as MS-209 (dofequidar fumarate), VX-710 and tariquidar (Minderman et al. 2004; Saeki et al. 2007; Patil et al. 2009), which are new ABC transporter inhibitors with good initial results. In breast cancer, Saeki et al. suggest that treatment with dofequidar plus cyclophosphamide, doxorubicin, and fluorouracil therapy resulted in possible clinical benefit for patients who had not received prior therapy, or who were premenopausal, or were stage IV at diagnosis with an intact primary tumor (Saeki et al. 2007). Tariquidar has been studied for the treatment of recurrent or metastatic ovarian, cervical, lung and kidney tumors. Nanoparticles encapsulating the combination of paclitaxel and tariquidar showed a significantly higher cytotoxicity in vitro and enhanced therapeutic efficacy of dual agent nanoparticles could be correlated with increased accumulation of paclitaxel in drug-resistant tumor cells (Patil et al. 2009). Other than in vitro studies, in vivo studies in a mouse model of drug-resistant tumor demonstrated significantly greater inhibition of tumor growth following treatment with dual agent nanoparticles encapsulating both paclitaxel and tariquidar when compared to single nanoparticles carrying only paclitaxel.

Another important strategy to target ABC transporters in cancer is to find new ways to avoid MDR proteins expression levels. Some studies have showed that specific signaling pathways could control or regulate the expression of these transporters, as shown by the regulation of MDR1 and ABCG2 by hedgehog signaling. Sims-Mourtada et al. (2007) have shown that inhibition of Hh signaling increases the response of cancer cells to different chemotherapies and also that Hh pathway activation induces chemoresistance in part by increasing drug efflux in an ABC transporter-dependent manner. Interestingly Hh signaling regulates the expression of the ABC transporter proteins multi-drug resistance protein-1 MDR1 and BCRP, and that targeted knockdown of these genes expression by siRNA partially reverses the Hh-induced chemoresistance.

8 Conclusions and Future Perspectives

Although the importance of CSC theory has been extensively demonstrated in the last decade, there are several challenges that restrict its translation in to the clinic. First, the pitfalls involving the correct characterization of BCSCs led to a misidentification and misuse of cellular markers such as CD44 and CD24, thus leading to a mischaracterization of aggressive non-stem cells as cancer stem cells. Another focal point is the characterization of BCSCs involved in drug resistance and disease recurrence and aggressiveness. Due to advances in both characterization and

functional details of pathophysiology of BCSCs, several studies showed insights that could drive future research regarding the potential of BCSCs as target cells for a better treatment of breast tumors.

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Chapter 8
Lung Cancer Stem Cells

Gavitt A. Woodard and David M. Jablons

Abstract Lung cancer remains the leading cause of cancer mortality and novel therapies are desperately needed to treat metastatic and recurrent disease. The cancer stem cell hypothesis is based on data that within each tumor there is a small sub-population of cancer stem cells that display the stem cell properties of self-renewal, pluripotency, a high proliferative capacity, and the ability to resist chemotherapy and radiation. These cancer stem cells are a likely cause of tumor resurgence after initial response to treatment and are an important therapeutic target. Distinct populations of epithelial cells in the airway and lung have been identified as the cells of origin for the major types of lung cancer: adenocarcinoma, squamous cell cancer, and small cell lung cancer. As we develop new therapies that target these cancer stem cell populations important work is underway to identify reliable cancer stem cell markers and to better understand the major pathways that fuel cancer stem cells. There is great interest in developing antibodies and small molecule inhibitors to the Wnt, Sonic Hedgehog, and Notch pathways to target cancer stem cells in lung and other malignancies, and multiple new drugs are in various stages of clinical trials. Lung cancer stem cells are a promising therapeutic target and important work remains to be done to better understand the role that lung cancer stem cells play in tumor development and recurrence.

Keywords Lung cancer • Lung cancer stem cells • Surface markers • Wnt • Sonic Hedgehog • Notch

1 Introduction: The Cancer Stem Cell Model in Lung Cancer

Lung cancer remains a highly aggressive cancer and is the leading cause of cancer-related mortality in the United States and worldwide with an overall 5 year survival rate of 19.3 % (Howlader et al. 2013). Only 20 % of lung cancer patients are

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surgical candidates at the time of presentation and 30–50 % of these early-stage tumors will recur following a complete surgical resection (Kelsey et al. 2009). Once patients have developed metastatic disease, only 15 % will be alive after 1 year and there are virtually no long term survivors (Groome et al. 2007). This highlights the importance of developing treatment strategies that target the mechanisms leading to tumor invasion and metastasis. For patients with more advanced disease, platinum based chemotherapy, targeted kinase inhibitors, and radiation can result in dramatic responses; however, almost all of these tumors recur within 2–3 years (Lin et al. 2014).

A stem cell is defined by high proliferative capacity, ability for self-renewal, and multipotency in producing daughter cells of varying types. The cancer stem cell (CSC) model is based on clinical and experimental data that a subpopulation of tumor cells displays stem cell like properties including the capacity for self-renewal, differentiation, and the ability to resist cell death from chemotherapy and radiation. Not all cancer cells posses these traits, nor do all cancer cells have the ability to generate a metastasis or a new tumor as cancer cells may be found circulating in the blood of patients who do not always develop metastases (Reya et al. 2001). CSCs have the unique ability to support new growth in xenograft models, whereas other cell populations from the same tumor are unable to repopulate a tumor in the same growth environment. The CSC model has mounting evidence in hematologic malignancies and solid tumors. CSC were first demonstrated in lung cancer in 1982 by Carney et al. who showed that a subpopulation of cells from adenocarcinoma and small cell lung cancer (SCLC) had stem cell-like properties, were able to form colonies in agar, and grow new tumors in athymic nude mice (Carney et al. 1982).

The CSCs give rise to highly proliferative progenitor cells which produce the differentiated cells that define the histologic type of lung cancer. Standard chemotherapy and radiation target the more rapidly dividing differentiated cells and can melt away the bulk of a tumor. However the CSCs divide at a lower rate and have additional mechanisms to resist chemotherapy. Treatment may result in a significant reduction in tumor bulk, but the remaining small number of CSCs has the capacity to eventually repopulate the tumor. Developing therapies that target these CSCs is crucial in preventing cancer recurrence.

2 Pulmonary Histology

In the tracheal and bronchial epithelia, endogenous stem cells in make up just 0.06–1.3 % of all proliferating cells, a smaller number than in the epithelia of the gut or skin where there is a much higher rate of cell turnover and epithelial repopulation (Snyder et al. 2009). The relative quiescence of pulmonary stem cells has made them more challenging to identify and isolate than stem cells in other tissues. Research using xenograft models of airway injury has identified cells throughout the airway that are responsible for repairing epithelial damage. By further investigating these cells, distinct cell populations in the lung have been identified that

display the properties of self-renewal, proliferation, and multipotency that define a stem cell.

2.1 Lung Development

Lung development in humans begins during the fourth week of embryogenesis with structures arising from the laryngotracheal groove. The trachea splits ventrally from the foregut by forming tracheoseophageal ridges which then fuse to create the tracheoseophageal septum. Caudal to this process the lung bud appears and divides into a right and left bronchial bud. In the fifth week the right and left primary bronchial buds divide into secondary buds, which will ultimately form the five lobes of the lungs, and then into tertiary buds which are the basis for the 19 lung segments in the fully developed lung. From this point through the sixteenth week, the lung will continue to grow and develop its major anatomic structures. Alveoli do not begin to form until after the 16th week when the bronchi become well vascularized and enlarged. The terminal airway structures continue to grow and mature until the 26th week of embryogenesis when the blood-air barrier is created. The alveoli saccules develop at the end of each terminal bronchiole and specialized alveolar cells for gas exchange and surfactant production develop (Schoenwolf et al. 2009).

The stem cell pathways Wingless type (Wnt), Sonic Hedgehog (Shh), and Notch, which will be discussed later, play important highly conserved roles in embryogenesis and in maintaining endogenous lung stem cells. During gestation Wnt regulates lung epithelial and mesenchymal development (Morrisey 2003). Mice knockouts have shown that specifically *Wnt-2*, *Wnt-5a*, and *Wnt-7b* are crucial for proper lung maturation (Yamaguchi et al. 1999; Shu et al. 2002). The critical role of the Shh pathway in lung development in mice has been extensively studied, but comparatively less is known about its role in human lung development. Zhang et al. (2012) demonstrated Shh in human lung development has many similarities with murine lung development. Shh is expressed in the developing lung epithelium, as are the Shh receptors Ptch1 and Smo and the Shh signaling effectors Gli1, Gli2, and Gli3. Notch signaling, which plays a role in cell fate decisions, is present early in development at the time of the lung epithelial buds (Tsao et al. 2008). These pathways are integral to normal lung development, cell maintenance, and injury repair, and represent important potential lung CSC targets.

2.2 Cell Diversity

The lung epithelium has many important functions including warming inspired air, performing gas exchange, and defending against pathogens. The epithelium is exposed to a number of insults during normal respiration. The crucial role of maintaining the integrity of the epithelium is performed by airway stem or progenitor

cells. The epithelium in the proximal portion of the upper airways and trachea is a pseudostratified epithelium consisting of ciliated, Clara, and goblet cells. More distally in the smaller airways and bronchioles the epithelial cells become more columnar. There is an increased number of Clara cells, with basal cells and rare neuroendocrine cells found at intervals between the columnar cells.

Within the terminal alveoli, the pneumocytes that comprise the alveolar wall are the alveolar type 1 (AT1) cells, alveolar type 2 (AT2) cells, and macrophages. The squamous AT1 cells are responsible for the structure of the alveolar wall and provide the surface for gas exchange. Cuboidal AT2 cells produce surfactant, a phospholipid and protein mixture which lowers the surface tension and facilitates gas exchange. These AT2 cells are responsible for repairing and repopulating the AT1 cells after injury (Desai et al. 2014).

2.3 Injury Response

During lung development, epithelial branching leads to distinct functional zones along the airway. Within each zone there is a unique cellular composition and set of local progenitor stem cells responsible for repopulating each area. These regional stem cells reside in discrete areas known as the stem cell niche within each portion of the airway. Recognizing these populations is important for CSC research as these endogenous stem cells may be the cells of origin in many cancers and there is insight to be gained from the pathways and mechanisms that confer stem cell properties.

Identifying the cells and stem cell niche in the lung has posed a challenge as the respiratory tract undergoes relatively slower cell turnover rates compared with other systems like the gastrointestinal tract and skin. Therefore the endogenous stem cells populations in the adult lung have been identified via a series of mouse injury models. In the trachea and upper airways the submucosal glands harbor the basal cells which repair and repopulate damaged tissue. A subpopulation of basal cells has been shown to behave like stem cells in response to injury. Borthwick et al. (2001) used the cell surface marker keratin-5 to label for a pluripotent population of tracheal basal cells. Later, Rock et al. (2009) confirmed this finding by labeling keratin-5 and using lineage tracing to demonstrate subsequently labeled Clara and ciliated cell populations in a steady state of airway maintenance, demonstrating that the basilar cells have an important role in tracheal and upper airway maintenance and repair. In addition there was an increase in the number of labeled cells following airway damage, suggesting that the basal cells were the progenitors of cells used for airway repair. In contrast, ciliated cells have been shown to be terminally differentiated and unable to self-renew (Rawlins et al. 2007). In addition to keratin-5, human lung basal cells can be purified using the surface markers ITGA6 and NGFR, and those purified cells are capable of self-renewal and generating luminal daughter cells in vitro (Rock et al. 2009). Collectively, these data suggest that basal cells act as the stem cell of the proximal upper airway.

More distally, the stem cell niches are the branch points of the smaller airways and the bronchoalvoelar duct junctions (BADJs) where the bronchi become alveoli. To identify the stem cells of the BADJ, many experiments have been performed to elucidate which cells display stem cell-like properties. Marked neuroepithelial bodies (NEBs) are found in the BADJ. The NEB consists of two cell types: "variant" Clara cells which express Clara cell secretory protein (CCSP) and pulmonary neuroendocrine cells which are marked by calcitonin gene-related peptide (CGRP) (Giangreco et al. 2007). Cells that express both surface markers CCSP and CGRP proliferate within the NEB during embryogenesis, airway maintenance, and repair. The "variant" Clara cells, defined by the surface protein CCSP, were identified by Reynolds et al. (2000) by exposing mice to naphthalene, a chemical that causes selective Clara cell death. In the presence of naphthalene Clara cells were destroyed, however "variant" Clara cells showed up-regulated activity and were able to subsequently repair airway damage (Giangreco et al. 2002).

AT2 cells produce surfactant and can therefore be recognized by secretory vesicles that containing surfactant protein C (SP-C) (Desai et al. 2014). At the BADJ, Giangreco et al. (2002) identified a population of bronchioalveolar stem cells which mark positive for CCSP and SP-C. These CCSP+ SP-C+ cells were shown to be the predominant proliferative cell population following bronchiolar damage and had the ability to differentiate into Clara, AT1, and AT2 cells. In addition, the CCSP+ cells at the BADJ retained their function even outside of the NEB microenvironment. There are likely multiple possible progenitor cell populations at the BADJ. Other interesting data has shown that SP-C negative cells are capable of regenerating AT2 cells following injury, indicating another potential alveolar progenitor stem cell population (Chapman et al. 2011).

Unfortunately, not all evidence on CCSP+ cells has been consistent. CCSP+ labeled populations have been shown to repopulate damaged AT2 cells following influenza or bleomycin-induced alveolar damage (Zheng et al. 2013) but not after naphthalene or oxygen exposure (Rawlins et al. 2009). Other cell populations may be active under these circumstances and more investigation is needed to identify additional repair mechanisms.

In mouse models, there are data that the important role of maintaining epithelial integrity is performed by committed Clara and AT2 progenitors with Clara cells repopulating the ciliated cell populations, and AT2 cells giving rise to lost AT1 cells in the alveoli (Rawlins et al. 2009; Evans et al. 1976). However, in humans data shows that the airway epithelium is maintained not by these specific subpopulations, but by a large number of progenitor basal cells which divide as necessary to maintain and repair the airway without pre-programmed stem cells (Teixeira et al. 2013).

These endogenous stem cell populations play a crucial role in repairing and maintaining airway epithelial integrity. In chronic lung disease compromise of the airway stem and progenitor cell populations is seen in chronic obstructive pulmonary disease and asthma (Staudt et al. 2014). Conversely, inappropriate up-regulation of these stem cells is implicated not only in lung cancer but in other diseases like

idiopathic pulmonary fibrosis where there are increases in the Wnt/β-catenin stem cell pathway (Chilosi et al. 2003).

3 Models of Cancer Development from Progenitor Stem Cells

There are two theories of tumorigenesis linking stem cells to cancer. In one theory endogenous stem cells are present long enough over a lifetime to accumulate a series of genetic mutations which ultimately become oncogenic and lead to development of a cancer. The other theory is based on the idea that cancers develop from a differentiated, restricted progenitor cell which through oncogenic mutations, acquires more mutations and ultimately these mutations confer the stem cell properties of self-renewal, pluripotency and immortality. There is evidence to support both theories and most likely tumors develop as a combination of these two mechanisms and continue to evolve with natural selection favoring the survival of cells with the most oncogenic, proliferative mutations.

In lung cancer this has been explored by attempting to link different histologic types of lung cancers with specific endogenous stem cell populations. Using mouse models of lung cancer, researchers have identified likely cells of origin and potential stem cells targets for the major histologic types of lung cancer. Different histologic types of lung cancer generally develop centrally or peripherally along the airway and each type of cancer shares characteristics with the lung stem cell population found within each of these anatomic areas (Giangreco et al. 2007).

3.1 Adenocarcinoma

Non-small cell lung cancer (NSCLC) is a broad grouping of primary lung tumors including adenocarcinoma, squamous cell carcinoma, and large cell neuroendocrine carcinoma which combined comprise 80 % of all lung and bronchus tumors (Howlader et al. 2013). Adenocarcinoma is the most common form of NSCLC and lung cancer overall, accounting for 38 % of all newly diagnosed lung cancers (Travis 2011). Recent advancements in targeted therapies for adenocarcinoma with specific tyrosine kinase inhibitors and targeted antibodies have led to modest improvements in survival times for certain subgroups of patients; however, additional therapeutic strategies are desperately needed (Yang et al. 2014; Rossi et al. 2014). In particular, therapies which target the quiescent and chemotherapy resistant stem cell population within each tumor would be a useful adjunct to current therapies that target more rapidly dividing cells.

In identifying the cells of origin in lung adenocarcinoma, much research has utilized *K-ras* mutated xenograft models. Approximately 25 % of human lung

adenocarcinomas have an activating *K-ras* mutation. These mutations are seen more commonly in smokers and are predictive of chemotherapy resistance and poor prognosis (Riely et al. 2008). Transgenic mouse models with activating *K-ras* mutations have been used to induce lung adenocarcinoma development, and used to identify the adenocarcinoma stem cells of origin. Xu et al. (2012) have demonstrated that lung hyperplasia, a precursor to cancer, originates from AT2 cells, terminal bronchial Clara cells, and putative bronchoalvoelar stem cells. However, only hyperplastic AT2 cells in the distal lung actually progress into lung adenocarcinoma. There is other evidence that Clara cells or AT2 cells are the originators of adenocarcinoma since lung adenocarcinomas frequently co-express the markers CCSP and SP-C which are co-expressed by Clara or AT2 cells (Giangreco et al. 2002). Based on current information, the stem cell origin of adenocarcinomas is mostly the Clara or AT2 cells found at the BADJ.

3.2 Squamous Cell Lung Cancer

The second most common type of lung cancer is squamous cell carcinoma (SCC) which accounts for 30 % of all NSCLC. SCC is recognized by the histologic characteristics of keratin pearls and intercellular bridges (Linnoila 1990). SCC in the lung is thought to develop through a process of dysplastic changes over several years similar to the way that squamous cell cervical cancer develops. The lung stem cell most likely linked to SCC is the basal cell since SCCs tend to develop in areas with the highest basal cell concentration at the submucosal gland duct junctions and at intracartilaginous borders. SCC likely occur as basal cell hyperplasia develops into metaplasia, to dysplasia, and ultimately to carcinoma in situ and invasive SCC disease (Jeremy George et al. 2007). Throughout this progression the squamous cells have been shown to maintain a basal cell phenotype and have persistent keratin-5 expression (Barth et al. 2000).

3.3 Small Cell Lung Cancer

Small cell lung cancer (SCLC) is distinct from NSCLC and is characterized by rapidly dividing cells, early development of widespread metastasis, and markedly worse survival outcomes (Elias 1997). Based on the most recent National Cancer Institute's data, SCLC comprises only 11 % of all new lung cancer diagnoses (Howlader et al. 2013). Outcomes in SCLC are so poor the disease is not staged by standard TNM criteria but by grouping patients into limited-stage and extensive-stage categories. Outcomes have remained poor over the past several decades with only 4.6 % of all patients remain alive 2 years following diagnosis. In the 40 % of patients that present with more favorable limited-stage disease there is still only a

10 % 5-year survival rate (Govindan et al. 2006). While most patients with SCLC will initially respond to chemotherapy and radiation, disease recurrence remains a major problem (Stupp et al. 2004).

SCLC has long been considered to arise from neuroendocrine cells as a more aggressive form of a carcinoid tumor of the lung. Phenotypically, SCLC cells have neuroendocrine characteristics and exhibit dense neurosecretory granules. They express the neural cell adhesion molecule synaptophysin and calcitonin gene-related peptide (CGRP). CGRP is also expressed by neuroendocrine cells in the NEB, implicating those neuroendocrine cells as the stem cells of origin for SCLC. Genetically, 70 % of SCLCs have a mutation or loss of heterozogosity in *Rb1* and *p53* (Meuwissen and Berns 2005). Sutherland et al. (2011) demonstrated that SCLC arises most frequently from the NEB when *Rb1* and *p53* are knocked out in Clara, AT2, and neuroendocrine cells of transgenic mice. Of those three cell types, neuroendocrine cells were the most easily transformed into SCLC and Clara cells were the most resistant. These data suggest that the SCLC cells of origin may be not only neuroendocrine cells, but a small number of AT2 and an even rarer population of Clara cells that also have the potential to develop into SCLC.

4 Identification of Lung Cancer Stem Cells

The ability to correctly identify and isolate CSCs is crucial for ongoing research and development of potential therapeutics. In order to classify the cell as a CSC, the isolated cells must display the properties of extensive proliferation and self-renewal in in vitro experiments. Thus far, most CSC research has used cells identified by surface proteins detected using flowcytometry, magnetic bead isolation, fluorescent protein tagging, and immunostaining.

4.1 Surface Markers

The best studied surface marker in lung cancer stem cells is CD133 (prominin-1 or AC133), which was first described in human hematopoietic stem cells (Miraglia et al. 1997). In lung cancer $CD133^+$ cells have been identified in both SCLC and NSCLC tumors. $CD133^+$ cells are rarely found in normal lung tissue but are seen more commonly in lung tissue in the process of regeneration. In both SCLC and NSCLC, $CD133^+$ cells have been shown to possess stem cell properties of pluripotency, self-renewal, and immortality. Eramo et al. (2008) showed that both SCLC and NSCLC $CD133^+$ cells were able to grow indefinitely in media in vitro. They also demonstrated that SCLC and NSCLC $CD133^+$ cells were able to generate phenotypically identical tumors in immunocompromised mice and were able to self-renew and generate unlimited progeny of non-tumorigenic cells in the same xenograft model. Bertolini et al. (2009) showed that injection of a purified pool of

$CD133^+$ cells, but not $CD133^-$ cells, into immunodeficient mice led to tumor development. And compared with $CD133^-$ cells, $CD133^+$ cells had higher expression of the genes involved in stemness, adhesion, motility, and drug efflux.

There is interesting data to suggest that $CD133^+$ cells are more resistant to chemotherapy and therefore can evade standard treatments and later repopulate tumor bulk as a mechanism for tumor recurrence. Bertolini et al. (2009) have also shown that cisplatin treatment can reduce the size of lung tumors in mice xenotransplanted with lung cancer stem cells. However the cells that remain following treatment are universally $CD133^+$ suggesting that the population of cells resistant to chemotherapy are the CSC. This may explain why following a complete radiographic remission patients will later present with tumor regrowth and widespread metastasis, produced by a handful of remaining CSC. In vitro treatment of lung cancer cells with cisplatin enriches for $CD133^+$ cells, and cisplatin treatment of lung cancer xenografts spares subpopulations of $CD133^+$ $ABCG^+$ cells and $CD133^+$ $CXCR4^+$ cells. $CD133^+$ cells express high levels of ABCG2, implying possible overlap with side population cells, as well as the embryonic stem cell markers Oct-4 and Nanog (Eramo et al. 2008). NSCLC patients whose cancers are $CD133^+$ have a tendency towards shorter progression-free survival after treatment with a platinum-containing chemotherapy regimen (Bertolini et al. 2009).

Similar evidence was provided by Levina et al. (2008) who showed that treatment of lung cancer cell lines with cisplatin, etoposide and doxorubicin enriched for $CD133^+$ expression and the side population phenotype. Following treatment the resulting cells were more tumorigenic when xenotransplanted into immunodeficient mice. Also of note, the cells that were able to survive chemotherapy contained two to threefold higher levels of human angiogenic and growth factors cytokines. These data indicate that traditional chemotherapy kills many cancer cells and shrinks tumor bulk but leaves behind a chemotherapy-resistant, aggressive CSC population. This suggests that chemotherapy in face selects for the survival of the most oncogenic cells with a higher expression of genes that promote tumor growth and metastasis.

While CD133 is a promising marker, not all human lung cancers have a population of $CD133^+$ cells (Bertolini et al. 2009) and there is data that $CD133^-$ cells from human lung cancer cell lines are also capable of producing tumors in a xenograft model (Meng et al. 2009). In addition, there has not yet been a consistently proven prognostic correlation between $CD133^+$ cells and survival (Salnikov et al. 2010). A recent meta-analysis of 13 different studies found that CD133 expression was not associated with disease free survival but was associated with shorter overall survival (Wang et al. 2014).

Another well studied stem cell surface marker is CD44, a transmembrane glycoprotein found in about half of NSCLC tumors and in particular in squamous cell NSCLC (Leung et al. 2010). Of note and unlike CD133, $CD44^+$ cells have not been observed in SCLC cell lines (Qiu et al. 2012). Like $CD133^+$ cells, $CD44^+$ cells are also enriched for stem-cell like properties. $CD44^+$ cells from lung cancer cell lines are able to initiate tumor growth in nude mice and perform in vivo differentiation by growing tumors containing both $CD44^+$ and $CD44^-$ cells. Isolated $CD44^+$ cells and

not CD44$^-$ cells express the pluripotency genes *OCT-4/POU5F1*, *NANOG*, and *SOX2*. CD44$^+$ cells also display resistance to cisplatin treatment in vitro with less apoptosis than CD44$^-$ cells (Leung et al. 2010).

As a prognostic marker, high CD44 expression in human NSCLC has been correlated with more advanced regional lymph node metastasis (Ko et al. 2011). However, there is mixed data that CD44$^+$ expression has prognostic significance. In squamous cell cancer, some studies have shown that CD44 expression is an independent marker for better overall survival in squamous cell lung cancer (Sterlacci et al. 2014) but others have shown that there is no correlation between CD44$^+$ cells and survival (Ko et al. 2011). There is also conflicting data regarding CD44 expressing adenocarcinomas, with some data that high CD44 expression is an independent negative prognostic marker (Ko et al. 2011) and another study which showed that patients with CD44$^+$ adenocarcinomas have longer overall survival (Leung et al. 2010).

CD166 is another marker that enriches for cells with stem-cell properties. It has been shown that in immunocompromised mice, transplantation of CD166$^+$ cells can create tumors with heterogeneous cell compositions that mirror that of the primary tumor. CD166 expression may be a poor prognostic indicator as CD166$^+$ cells overexpress glycine decarboxylase, which has been shown to correlate with worse survival prognosis in NSCLC (Zhang et al. 2012).

Urokinase-type plasminogen activator (uPA) and its receptor uPAR/CD87 are regulators of extracellular matrix degradation and are important for cell migration and invasion. In cancer uPA it is a strong predictor of poor outcomes. In SCLC cell lines, uPAR$^+$ cells have been shown to have enhanced clonogenic activity and multidrug resistance in vitro (Gutova et al. 2007) and in vivo are tumorigenic in athymic nude mice (Qiu et al. 2012). Cancer cells that are uPAR$^+$ also co-express other CSC surface markers including CD133, CD44, and MDR1 (Qiu et al. 2012; Gutova et al. 2007).

4.2 Side Population Cells

In addition to surface markers, CSC can be identified by functional attributes. In the course of studying lung cancer cells with flowcytometry, an incidentally found side population (SP) phenotype of cancer cells was repeatedly seen on flowcytometry (Fig. 8.1). This SP cell population has an ATP-binding cassette (ABC) family transporter cell membrane protein which pumps out the fluorescent nuclear dye Hoechst 33342. By pumping out the nuclear dye these SP cells appear as a side population to the reminder of the tumor on cell sorting and can be isolated from the rest of the tumor. SP has been detected in studies of SCLC and NSCLC cell lines. In squamous cell cancer SP cells have higher rates of proliferation and greater clonogenic ability in vitro, and a significantly lower concentration of isolated SP cells compared with non-SP cells are capable of producing tumors when xenotransplanted into immunocompromised mice (Loebinger et al. 2008). Isolated SP cells are multipotent and

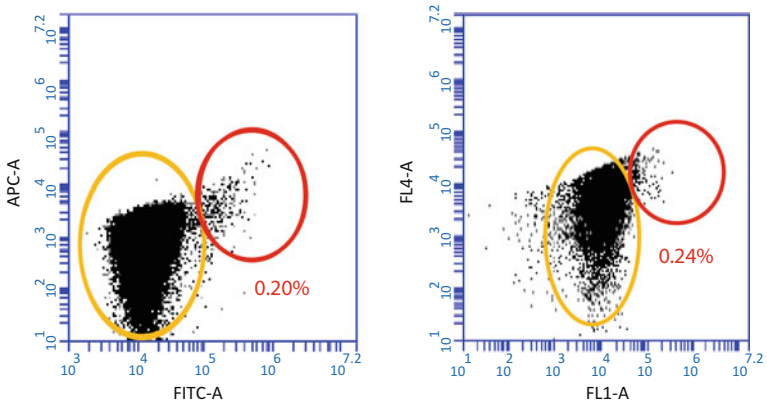

Fig. 8.1 *Side population cells*. The cancer stem cell side population cells have an ABC transporter cell membrane protein which pumps out the fluorescent nuclear dye Hoechst 33342 and separates these rare cancer stem cells (*red circle*) from the majority of the tumor cell population (*yellow circle*) on flow cytometry. These cells make up less than 1 % of all tumor cells. Side population cells have been identified from both lung cancer cell lines (*left*) and from fresh lung cancer surgical specimens (*right*).

produce both SP and non-SP cells in culture. It has been shown in vitro invasion assays that SP cells also have a higher potential for invasiveness than non-SP cells (Ho et al. 2007).

SP cells are significantly more resistant to chemotherapy in assays in vitro and are more capable of maintaining a large colon formation than non-SP cells in the setting of chemotherapy (Ho et al. 2007). The increased ABC transporters that remove Hoechst dye from SP cells and provide a mechanism for the SP cell's relative chemoresistance, and this is further proven by data that SP cells can be sensitized to chemotherapy by blocking the ABC transporter with verapamil (Loebinger et al. 2008). Studies of SP cells suggest that they are more quiescent than other tumor cells as they have higher levels of telomerase and have less mini-chromosome maintenance 7, a marker of proliferation (Ho et al. 2007).

4.3 Aldehyde Dehydrogenase

Aldehyde dehydrogenase (ALDH) is involved in early stem cell development where it oxidizes retinol to retinoic acid (Chute et al. 2006). ALDH has been recognized as a stem cell marker in multiple studies. Jiang et al. (2009) showed that ALDH1$^+$ cells were able to self-renew, had proliferative tumorigenic potential in in vivo studies, and showed resistance to chemotherapy. ALDH1$^+$ cells have been shown to overlap with cells that express the stem cell marker CD133 but there is limited other data to suggest that these stem cells markers occur within the same cell population. ALDH activity is associated in particular with squamous histology (Moreb et al. 2007).

High ALDH1 protein expression has been associated with poor prognosis in early NSCLC (Jiang et al. 2009; Sullivan et al. 2010). This may be partially explained by work by Moreb et al. (2008) who showed that if ALDH protein was inhibited by siRNA, tumor cells had a reduced ability to proliferate and migrate in vitro.

A single, universal marker for lung cancer stem cells has yet to be identified. The current surface makers, SP cell type, and ALDH lack sensitivity and specificity for consistently selecting cells with stem cell properties. Akunuru et al. (2012) have demonstrated that non-SP, $CD133^-$, $ALDH^-$ cells can produce SP, $CD133^+$, and $ALDH^+$ cells in culture, exhibiting the phenotypic switching from a non-cancer stem cell type to a cancer stem cell phenotype. This study and the relative paucity of data showing overlap between the currently identified stem cell surface markers cast some doubt over the accuracy of our current methods to identify stem cell populations and better markers for lung cancer stem cells are needed.

5 Stem Cell Pathways and Molecular Targets

The properties which enable endogenous stem cells to perform their crucial role in normal tissue repair also serve as protective mechanisms against cell death. Stem cells have ABC transporter proteins which lead to rapid toxin and drug efflux as well as high levels of anti-apoptotic proteins. CSCs share these properties which confer an increased resistance to traditional chemotherapy and radiation. In addition, most chemotherapy agents work by targeting rapidly proliferating cells and for this reason may be less effective against the CSCs which are more quiescent in nature. Without eliminating the CSCs, these cells remain in the body as a small but potent tumor reservoir that can ultimately repopulate a cancer recurrence.

For this reason CSCs are important, if not the most important therapeutic target and research into treatments which target the CSC specific signaling pathways such as Wingless type (Wnt), Sonic Hedgehog (Shh), and Notch is of interest in lung and other cancers (Takebe and Ivy 2010). Targeted therapies to block these pathways poses a challenge as these are the same signaling pathways used by normal proliferating cells and there are protective cross-talk mechanisms between pathways that preserve their important role.

5.1 Wnt Pathway

Wingless type (Wnt) glycoproteins are a highly conserved family of 19 secreted signaling molecules that bind to cell surface receptors and regulate downstream gene expression (Angers and Moon 2009). The Wnt pathway plays a crucial role in embryogenesis, lung development, and endogenous stem cell regulation. During embryogenesis and development Wnt proteins control cell fate determination and

direct the development of the cardiovascular, pulmonary, renal, and central nervous systems (Grigoryan et al. 2008). Later in life the Wnt pathway regulates tissue self-renewal, including the renewal of hair follicles, intestinal crypts, and bone growth plates (Clevers 2006; Andrade et al. 2007). Deregulated Wnt signaling has been shown in a large variety of cancers including hepatocellular carcinoma, hepatoblastoma, colorectal cancer, acute and chronic myelogenous leukemia, multiple myeloma, gastric cancer, Wilms' tumor, and NSCLC (He et al. 2005a). Wnt signaling has been shown to promote stem cell self-renewal in hematopoietic stem cells (Reya et al. 2003). Wnt pathway activation, specifically β-catenin signaling, has been shown to be required for cancer stem cells to maintain their tumorigenic potential (Malanchi et al. 2008). The Wnt pathway is therefore an important potential target to eliminate or inhibit cancer stem cell growth.

In the canonical pathway, a Wnt ligand binds to a Frizzled (Fz) receptor or an LDL-receptor related protein (LRP) on the cell surface. This activates one of three intracellular Dishevelled (Dvl) proteins, Dvl-1, Dvl-2, or Dvl-3, which then inhibit glycogen synthase kinase-3β (GSK-3β). When Dvl inhibits GSK-3β, it prevents GSK-3β from phosphorylating β-catenin. In the presence of Wnt signaling free β-catenin stabilizes, accumulates in the cytosol, and ultimately translocates to the nucleus. There β-catenin interacts with either p300 or cyclic AMP response element-binding protein (CBP) and with members of the T-cell factor-lymphocyte enhancer factor (TCF/LEF) family of transcriptional factors which activate target genes including *Myc, Cyclin D1, TCF-1, PPAR-δ, MMP-7, Axin-2, CD44, Cox2* (Takebe et al. 2011; Mazieres et al. 2005). On the cell's surface 10 different Fz receptors have been identified providing multiple Wnt-Fz receptor combinations that can subtlety modify the downstream effects of Wnt (Schulte and Bryja 2007). Wnt has also been shown to be active in at least two noncanonical pathways by activating calmodulin kinase II and protein kinase C in the Wnt/Ca^{++} pathway and by activating Jun N-terminal kinase in the planar cell polarity pathway (Veeman et al. 2003).

In lung cancer multiple mechanisms of increased Wnt activation have been identified. Uematsu et al. (2003a) demonstrated Dvl overexpression as one mechanism by which the Wnt pathway can be activated in NSCLC. Another studies showed that Wnt-1 and Wnt-2 are overexpressed in both NSCLC cell lines and in primary tumor tissue (He et al. 2004; You et al. 2004b). Other Wnt proteins such as Wnt-7a and Wnt-5a appear to behave as tumor suppressors. Wnt-7a is down-regulated in most lung cancer cell lines and primary tumor samples (Calvo et al. 2000). During development Wnt-7a functions via a non-canonical β-catenin independent pathway in developing human limbs (Kengaku et al. 1998). In lung cancer Wnt-7a appears to activate the canonical Wnt pathway, but does not directly target TCF-LEF transcriptional activity. It has been shown to positively regulate the epithelial-mesenchymal transition (EMT) marker E-cadherin expression in lung cancer cells (Ohira et al. 2003). Like Wnt-7a, Wnt-5a activates a non-canonical pathway in development, the Wnt/Ca^{++} pathway. In some cancers Wnt-5a is up-regulated and associated with increased tumor invasion, including the development of lung metastasis in sarcoma (Saitoh et al. 2002; Nakano et al. 2003). However, in hematopoietic malignancies Wnt-5a acts as a tumor suppressor and its role in primary NSCLC has not been studied.

The expression of downstream proteins in the canonical Wnt pathway is an area of ongoing research in NSCLC. Dvl-3 is overexpressed in 75 % of NSCLC tumor samples compared with autologous matched normal tissues from the same patient. Deletion of the PDZ protein binging domain of Dvl blocks Dvl activity and suppresses tumorigenesis in pleural malignant mesothelioma (Uematsu et al. 2003b). Data regarding β-catenin are more controversial. Mutations in the β-catenin gene are rare in lung cancer cell lines and in primary lung tumor tissue (Sunaga et al. 2001; Shigemitsu et al. 2001; Ueda et al. 2001). In NSCLC increased expression of β-catenin is associated with a high proliferative index but is unexpectedly associated with a better prognosis (Hommura et al. 2002). Other independent studies have corroborated this finding by showing that reduced β-catenin expression is associated with a better lung adenocarcinoma prognosis (Retera et al. 1998; Kase et al. 2000). These data may reflect β-catenin's involvement in the Wnt pathway and as a cadherin-mediated cell adhesion component implying a complex, multifacited role in NSCLC which is not yet fully understood (Barker et al. 2000).

Given the importance of the Wnt pathway in maintaining cancer stem cells there are a number of experimental agents in development to inhibit Wnt signaling with promising results. In vitro apoptosis can be induced if either Wnt-1 or Wnt-2 is inhibited by siRNA or a monoclonal antibody. Extracellular Wnt inhibition with monoclonal antibodies to Wnt and the Fz receptor has shown antitumor activity in vitro (He et al. 2005b; You et al. 2004a). And in vivo anti-Wnt-1 and anti-Wnt-2 monoclonal antibodies are also able to suppress tumor grown in a mouse model (He et al. 2004; You et al. 2004b). These results are promising but these monoclonal antibodies have not yet been tested in humans.

The highly conserved gene *Wnt inhibitory factor-1 (WIF-1)* has been shown to be down regulated in several cancers including prostate, breast, bladder, and lung (Wissmann et al. 2003). Mazieres et al. demonstrated that WIF-1 expression is down-regulated in 83 % of human NSCLC tumor specimens and proposed a mechanism of hypermethylation of CpG islands in the functional *WIF-1* promoter region (Mazieres et al. 2004).

Endogenous secreted frizzled-related proteins (sFRP) modulate *Wnt* signaling by competing with Wnt ligand binding to the Fz receptors and have been shown to be down-regulated in colon, gastric, and breast cancer. In lung cancer, Lee et al. (2004) demonstrated that sFRP are down-regulated in NSCLC and mesothelioma cell lines and that 80 % of mesothelioma tissue specimens have hypermethylation of the sFRP gene promoter. Dvl is another important therapeutic target for Wnt pathway inhibition. In lung cancer cell lines, targeted inhibition of Dvl-1, Dvl-2, or Dvl-3 decreases β-catenin expression, decreases TCF-dependent gene transcription, and inhibits tumor cell growth (Uematsu et al. 2003a).

IGC-001 (Institute for Chemical Genomics) is a small molecule that interrupts β-catenin binding to transcriptional cofactor CBP. In colon cancer cell lines ICG-001 results in apoptosis in cancer cells but spares the normal colon epithelial cells (Emami et al. 2004). Intracellular inhibitors NSC668036 (Sigma-Aldrich) and FJ9 are two other compounds in development which target the PDZ domain of Dvl and

inhibit both the canonical and non-canonical Wnt pathways (Fujii et al. 2007; Shan et al. 2005). Chen et al. have identified two additional small molecule inhibitors that block the Wnt pathway in vivo via different mechanisms. One small molecular is a membrane-bound acyltransferse small molecule Porcupine inhibitor, which is essential for Wnt synthesis, and the other small molecule inhibits the destruction of Axin, which suppresses Wnt activity (Chen et al. 2009).

5.2 Sonic Hedgehog Pathway

The Sonic Hedgehog (Shh) pathway is best known for its role in embryogenesis where it controls the migration, polarity, differentiation, proliferation, and transformation of progenitor cells (Varjosalo and Taipale 2008). If unregulated, thoses cellular processes also give the Shh pathway a significant role in carcinogenesis and the transformation of adult stem cells into CSCs. Activated Shh has been implicated in tumorigenesis and metastasis in multiple types of cancers including lung, brain, breast, prostate, and skin. In the canonical Shh pathway, the absence of the Shh ligand leads the transmembrane receptor Patched (Ptch) to inhibit the transmembrane receptor Smoothened (Smo). Inhibited Smo causes cleavage of Gli to the N-terminal repressor form. Therefore when Shh binds to Ptch, the inhibitory effect on Smo is released and active full length Gli is transported into the nucleus and Gli1, Gli2, and Gli3 transcription factors activates transcription of Gli-dependent target genes such as *Gli1*, *Ptch1*, *cyclinD1* and *Wnt* (Hooper and Scott 2005; Huangfu and Anderson 2006; Altaba et al. 2007; Mullor et al. 2001). Gli1 activates Shh target genes, Gli2 has a role in both gene activation and repression and Gli3 represses target gene transcription. The balance between activation and repression by the three forms of Gli appears to control Shh downstream signaling (Altaba et al. 2007).

In addition to the Shh pathway, non-canonical Gli activation independent of Shh, has been shown in many cancer cells types, (Lauth and Toftgard 2007; Mimeault and Batra 2010) and there is evidence for Gli activation independent of Shh, stimulated by other oncogenic signaling pathways such as transforming growth factor β (TGF-β), epidermal growth factor receptor (EGFR), RAS and AKT/PI3K pathways (Guo and Wang 2009; Schnidar et al. 2009; Pasca di Magliano et al. 2006; Stecca et al. 2007). As Gli transcription factors constitute the final effectors of the Shh pathway and are implicated in multiple other oncogenic signaling pathways, they represent an important downstream target for potential cancer therapeutics (Lauth and Toftgard 2007).

The Shh pathway contains many potential CSC therapeutic targets and drug development is an area of active research. The first Shh pathway inhibitor identified was cyclopamine (11-deoxojervine), a plant-derived steroidal alkaloid that binds to and deactivates Smo (Taipale et al. 2000). Park et al. (2011) have shown that Shh signaling is involved in SCLC development in genetically engineered mice and that

Shh inhibition can help prevent tumor recurrence. It has also been shown that inhibition of Shh signaling in SCLC with the Smo antagonist cyclopamine leads to loss of tumorigenicity (Watkins et al. 2003). Cyclopamine remains the only naturally derived compound but there are a growing number of synthetic small molecules designed to inhibit the Shh pathway at different points.

Vismodegib (GDC-0449, Genentech) is a Smo inhibitor approved by the U.S. Food and Drug Administration to treat adult patients with basal cell carcinoma (Ng and Curran 2011; LoRusso et al. 2011; Sekulic et al. 2012; Dlugosz et al. 2012). Response rates in the phase I clinical trial in metastatic or locally advanced basal cell cancer were encouraging, with over half of patients having at least a partial response. Common side effects of vismodegib include dysgeusia, hair loss, nausea, vomiting, anorexia, dyspepsia, weight loss, hyponatremia, and fatigue. A quarter of patients experienced adverse events including fatigue, hyponatremia, muscle spasm and atrial fibrillation and only one of 33 paitents developed a major dose limiting toxicity of grade 3 lymphopenia (Von Hoff et al. 2009). Vismodegib is currently being investigated in clinical trials to treat other types of cancer including ovarian, pancreatic, colorectal, and lung cancer due to its ability to selectively target Shh signaling (Ng and Curran 2011; Agarwal et al. 2011). In SCLC vismodegib is in clinical trials in combination with cisplatin and etoposide (ClinicalTrials.gov 2014).

Another small molecule Smo inhibitor is BMS-833923, XL139 (Bristol-Myers Squibb). BMS-833923 has completed phase I clinical trials in combination with carboplatin and etoposide as a treatment for SCLC. It is also being tested as part of a multidrug regimen in multiple myeloma and metastatic gastric and esophageal cancers (ClinicalTrials.gov 2014). Infinity Pharmaceuticals has developed a cyclopamine-derived inhibitor Shh inhibitor IPI-926 (Infinity Pharmaceuticals) which is in clinical trials for advanced-stage solid tumors and metastatic pancreatic cancer. Early clinical trial data in patients with basal cell carcinoma showed that IPI-926 is well tolerated and nearly a third of patients experienced a partial or complete clinical response (Jimeno et al. 2013). Multiple phase 2 trials are currently underway testing IPI-926 alone or in combination with other agents in for a myriad of advanced-stage malignancies (ClinicalTrials.gov 2014). In addition to the direct effect on Gli transcription factors there is emerging evidence that Shh signals have some control over the architecture of the stromal microenvironment. In a mouse model of pancreatic cancer IPI-926 improved access of chemotherapy agents potentially via this mechanism (Olive et al. 2009).

Other potential emerging therapies which have shown effects in in vitro testing include Robotnikinin, a small molecule that binds to extracellular Shh, and small synthetic molecules called Hedgehog Protein Inhibitors (HPI) 1–4 which inhibit downstream Gli activation through different mechanisms (Stanton et al. 2009). HPI-1 has been shown to inhibit activation of Gli1 and Gli2, HPI-2 and HPI-3 both inhibit Gli2, and HPI-4 inhibits formation of cilia when Smo is active and therefore prevents activation of Gli transcription factors (Hyman et al. 2009). Ongoing investigation into Shh is likely to elucidate other mechanisms by which this pathway drives cancer cell growth and uncover additional therapeutic targets.

5.3 Notch Pathway

The Notch pathway regulates cellular proliferation and differentiation via cell-to-cell communication and has a highly conserved role in determining cell fate during embroygenesis. It also plays a critical role in cellular proliferation, differentiation, apoptosis, hematopoiesis, breast development, colorectal epithelial maturation, immune regulation, and neural stem cell survival (Artavanis-Tsakonas et al. 1999).

When a Notch ligand pairs with a receptor on an adjacent cell it results in a coordinated cell-to-cell communication. In mammals, the membrane-bound Notch ligands are either Delta-like ligands 1, 3, and 4, or Jagged ligands 1 and 2 which are structurally distinct. These membrane ligands interact with four transmembrane, heterodimer Notch receptors 1, 2, 3, and 4 which contain multiple epidermal growth factor-like domains (EGF). The affinity of the ligand for the receptor depends on the EGF domain fucosylation by Fringe proteins Lunatic, Radical, and Maniac (Takebe et al. 2011). After Notch ligand-receptor binding the receptor undergoes a conformational change that exposes a site to proteolytic cleavage by metalloprotease which releases an extracellular fragment and cleavage by **γ**–secretase which releases an active Notch intracellular domain (NICD) fragment into the cytoplasm (Gordon et al. 2007). NICD then modulates Notch-specific gene expression by undergoing nuclear translocation and binding to the translocation initiation complex.

In endogenous stem cells, activated Notch signaling guides asymmetric cell division and retains stem cell viability (Artavanis-Tsakonas et al. 1999). In the healthy mouse lung, suppression of Notch signaling by knocking out *hairy and enhancer of split 1 (Hes1)*, increases the number of cells which differentiate into neuroendocrine cells and decreases the number of cells which become Clara cells (Ito et al. 2000). Constitutive activation of Notch in mice leads to delayed differentiation and accumulation of distal airway stem cells (Dang et al. 2003).

In human lung cancer cell lines, Chen et al. (1997) have shown elevated levels of Notch transcripts and Westhoff et al. (2009) reported possible oncogenic mutations in Notch1 receptor in NSCLC. However, there is other data to show that Notch may have a tumor suppressor effect in squamous epithelial in mice and in human myeloid cancers (Nicolas et al. 2003; Klinakis et al. 2011). These data that the Notch pathway can play both an oncogenic and tumor suppressor role suggest that the Notch pathway is complex. While further investigation is needed to better understand its role, Notch signaling remains an attractive potential therapeutic target.

Research by Moreb et al. (2008) demonstrated that $ALDH1^+$ cells express Notch pathway transcripts and that inhibition of the Notch pathway with γ–secretase inhibitors led to a reduction in $ALDH1^+$ cells. Osanyingbemi-Obidi et al. (2011) showed inhibiting Notch3 with γ–secretase suppressed clonogenic survival in cell lines and that this clonogenic survival could be restored by reintroducing the Notch3 receptor domain. γ–secretase inhibitors decrease tumor growth and the number of $CD133^+$ glioma stem cells in human glioma xenografts (Fan et al. 2006). Early studies of γ–secretase inhibitors in rodent models showed excessive toxicity and

therefore researchers are currently working to develop more specific inhibitors of the Notch transcriptional complex (Imbimbo 2008).

There are clinical trials underway to test novel Notch inhibitors in many malignancies including NSCLC (ClinicalTrials.gov 2014). The primary focus thus far has been on inhibiting γ–secretase mediated Notch cleavage. MK0752 (Merck) is a γ–secretase inhibitor which has been tested in the treatment of T-cell acute lymphoblastic leukemia. It was found to have dose limiting toxicity of gastrointestinal globet cell hyperplasia and secretory diarrhea, however these toxic effects have been shown to be reduced in a mouse model with co-administration of glucocorticoids (Real and Ferrando 2009).

5.4 Transcription Factors

In addition to the central role that the Wnt, Shh, and Notch pathways play in lung cancer stem cells, many niche factors and interactions with other signaling pathways have been shown to play an important part in maintaining a cancer stem cell phenotype. The transforming growth factor-β (TGF-β) family of cytokines has been shown along with Wnt, Shh, and Notch to induce EMT, the complex process by which cells down-regulate E-cadherin, lose their adhesive properties and cell polarity, and gain invasive and migratory properties (Massague 2008). The EMT process and loss of E-cadherin allows some cancer stem cells to become metastatic and has been associated with tumor metastasis and poor prognosis (Kim et al. 2009; Mareel et al. 1997). TGF-β interaction maintains stem cell characteristics in cells which have undergone EMT and may be a therapeutic target in eliminating metastatic cancer stem cells (Bailey et al. 2007).

Another transcription factor which has been shown to play a role in cancer stem cells is Octamer-binding transcription factor 4 (Oct-4). Oct-4 is a homebox transcription factor that is crucial for embryonic stem cell self-renewal along with Nanog and Sox2. There is also mounting evidence for its use as a stem cell marker. In NSCLC there is data to shows that Oct-4 regulates stem cell activity in $CD133^+$ cells (Chen et al. 2008). Ectopic expression of Oct-4 and Nanog, another homebox transcription factor, increases the percentage of lung adenocarcinoma cells that are $CD133^+$, enhances drug resistance, and promotes epithelial-mesenchymal transformation. Oct-4 is present in high grade tumors and is a negative prognostic marker of lung adenocarcinoma survival (Chiou et al. 2010). Chen et al. (2008) demonstrated that siRNA knockdown of Oct-4 reduces clonogenicity and increases sensitivity to chemotherapy in $CD133^+$ cells. CSCs maintain their stem cell properties by up-regulating certain highly conserved cell development and fate determination pathways. Inhibiting these pathways with novel therapies is a highly promising area of research. Chemotherapy that targets the resistant CSC population at the core of a tumor would be incredibly useful in the treatment of lung cancer and other malignancies.

6 Conclusion

The treatment of lung cancer remains challenging with dismal survival outcomes over the past several decades despite advances in chemotherapy and medical care. Lung cancer stem cells are the crucial target in developing new therapeutic strategies. The combined stochastic model of cancer stem cells explains mechanisms of tumor development from local endogenous stem cell populations within the airway, and provides mechanisms for tumor metastasis and recurrence. Within each lung cancer resides a small population of cancer stem cells that maintain the properties of self-renewal, pluripotency, immortality, and chemotherapy resistance. These lung cancer stem cells produce large numbers of progeny cells that comprise the bulk of a tumor. Current lung cancer chemotherapy eliminates the non-stem tumor cells and generates radiographic responses. However, CSCs possess mechanisms that prevent apoptosis and have been shown to survive these standard treatments. The remaining small number of potent lung CSCs over time repopulates a tumor leading to cancer recurrence with cells that have been selected for a phenotype that is even more oncogenic, treatment resistant, and aggressive.

There are significant challenges in developing treatments that target cancer stem cells. The first barrier is accurately identifying and successfully isolating lung cancer stem cells from human cancers into an in vitro research environment. Great progress has been made in identifying surface markers and sorting methods to isolate subpopulations of cells such as $CD133^+$, $CD44^+$ and the SP cells that exhibit stem-cell properties. However no universal stem cell marker has been identified and the variety of stem cell markers that have been discovered are not consistently co-expressed on the same cell.

As we continue to work towards improved methods of cancer stem cell identification there has been promising early work in exploring stem cell signaling pathways and discovering possible therapeutic targets. Wnt, Shh, and Notch signaling pathways all play important roles in embryogenesis and have been shown to be crucial regulators of cancer stem cell activities. Developing therapies that inhibit these cancer stem cells pathways remains tricky as they are highly conserved and used by endogenous stem cells in their normal function of tissue maintenance and repair. Multiple Wnt, Shh, and Notch inhibitors are in various stages of development. The Shh pathway Smo inhibitor vismodegib (Genentech) is already approved for metastatic or recurrent locally advanced basal cell carcinoma with further studies currently underway to expand its application to multiple other solid tumors, including a trial as a combined therapy in lung cancer with cisplatin and etoposide. As research into cancer stem cell pathways and potential drug targets continues to expand we can expect to see multiple new agents designed to target lung cancer stem cells. Understanding and eliminating cancer stem cells remains a promising and exciting area of research and a crucial component in the future treatment of lung cancer.

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Chapter 9
Colorectal Cancer Stem Cells

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Abstract Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer deaths in the United States with over 50,000 deaths per year. The sporadic colorectal cancer, which occurs in ~80 % of the patients, is an age-related disease, the incidence of which rises dramatically after 50 years of age. According to stochastic model of sporadic cancer, it was thought that all cancer cells that possess driver mutation(s) will lead to the process of carcinogenesis. However, in recent years, numerous studies have appeared to challenge the stochastic model. It is becoming increasingly accepted that not all, but only a small subpopulation of pluripotent self-renewing tumor cells that are termed as cancer stem cells (CSC) play a determinant role in the development and progression of many malignancies, including colorectal cancer. The focus of this book chapter is to briefly describe the role of cancer stem cells in recurrence of colorectal cancer, which leads to metastasis and remains a major clinical challenge. Although the underlying biochemical and molecular events leading to recurrence of various malignancies are not fully understood, CSCs that have been shown to be resistant to conventional chemotherapy play pivotal role in these processes. While the origin of CSCs is not fully known they are thought to be derived from mutations in normal stem, progenitor or differentiated cells. Despite recent advances in medicine, nearly 50 % of the patients develop recurrence of colon tumor that is highly enriched in CSCs. Unfortunately the conventional chemotherapy has shown limited success in treating recurrent cancer. This underscores the need for development of novel treatment strategies for recurrent colon cancer by targeting CSCs. Attempts are being made to target CSCs utilizing combination of chemotherapy and specific inhibitors of growth factor receptors or signal transduction. In addition, efforts have also been made to utilize non-toxic natural agent(s), either alone or in combination with conventional chemotherapy.

Keywords Colorectal cancer • Stem cells • Cancer metastasis • Drug resistance

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1 An Overview of Colorectal Cancer Biology and Pathology

Colorectal carcinomas are one of the most frequent neoplasms in the Western society. It is the second leading cause of cancer-related deaths in the United States. The American Cancer Society estimates about 140,000 new cases of colorectal cancer in the United States for 2014 with approximately 50,310 deaths due to this disease. Overall, the lifetime risk of developing colorectal cancer is about 1 in 20 (5 %) and can be graded into well, moderately and poorly differentiated lesions. Diet and lifestyle as well as inherited and somatic mutations are the contributing factors in CRC. The progression of colorectal neoplasms involves uncontrolled epithelial cell replication, continuation into formation of adenomas of various dimensions and eventually evolving into malignancy. This process has been termed the polyp-carcinoma sequence and the transformation from the initial events to an invasive carcinoma takes about 8–12 years. In the earliest phases of colorectal tumorigenesis, a disorder of cell replication is initiated in the normal mucosa, which is associated with clusters of enlarged crypts [aberrant crypts] having abnormal proliferative, biochemical and biomolecular characteristics. A well demarcated mass of epithelial dysplasia with uncontrolled crypt cell division is termed as an adenomatous polyp. An adenoma can be considered malignant, when neoplastic cells pass through the muscularis mucosae and infiltrate the submucosa. Hyperplastic polyps, serrated adenomas, flat adenomas, hamartomatous polyps, and inflammatory polyps are some other types of polypoid lesions beside adenomas. Thus colorectal tumors cover a wide range of premalignant and malignant lesions. The pathology of colorectal cancer has been reviewed in details earlier (Ponz de Leon and Di Gregorio 2001).

The focus of this chapter is on the sporadic colorectal cancers, which constitute 80–85 % of CRC and arise as a consequence of progressive genetic and epigenetic alterations that drive the transformation and progression of normal colonic epithelial cells to cancer. Fearon and Vogelstein presented a model for genetic alterations necessary for different stages of colorectal tumorigenesis (Fearon and Vogelstein 1990). According to this model, (a) colorectal cancer arises as result of genetic mutations in tumor suppressor genes and oncogenes; (b) mutations in at least 4–5 genes are required for the formation of a malignant tumor; and (c) total accumulation of changes rather than their sequence is important for the transformation. The molecular and genetic events leading to CRC have been reviewed in details earlier (Fearon 2011). A brief description of the transforming events is provided here. The first and most important mutation in the earliest adenomas leads to truncation of the APC (adenomatous polyposis coli) tumor suppressor protein. According to Kinzler and Vogelstein (Kinzler and Vogelstein 1996), APC plays a gatekeeper role in the normal colorectal epithelial cells maintaining a constant cell number; mutations in this gatekeeper gene lead to a permanent imbalance of cell division over cell death. *APC* encodes a 300 kDa protein that may regulate cell-cell adhesion, cell migration, chromosomal segregation and apoptosis in colonic crypt (Polakis 2007; Aoki and Taketo 2007; Brocardo and Henderson 2008). The best established function of

mutated (inactivated) APC protein is to partner with β-catenin leading to activation and up-regulation of oncogenes such as cyclin D1 and c-myc as well as many other tumor associated genes (Aoki and Taketo 2007; Polakis 2007).

The second most common mutations observed in almost 40 % of colon carcinomas are in the *KRAS* gene (Malumbres and Barbacid 2003). These mutations contribute to colorectal adenoma development, but are not required for the initiation event. Ras proteins regulate several downstream signaling cascades including mitogen activated protein kinase (MAPK) and PI3K pathways. Moreover, mutations in PI3K gene *PIK3CA* were reported in approximately 15–25 % of CRCs (Wood et al. 2007; Samuels et al. 2004). In addition, loss of PTEN expression is observed in about 15–20 % of CRC, although the somatic mutations in *PTEN* are found only in 10 % CRC. The above mutations result in activation of Akt and mTOR pathways, which regulate apoptosis and nutrient availability during cellular growth.

The mutations in *p53* gene are associated with adenoma-carcinoma transition (Baker et al. 1990). The p53 protein regulates the transcription of several proteins such as p21, PUMA, BAX and MDM2 that serve as cell cycle checkpoint, promote apoptosis and restrict angiogenesis. It is suggested that mutation in *p53* facilitates the growth and acquisition of invasive properties in the adenoma cells, which otherwise will be severely limited due to the stresses of rapid tumor growth. *p53* mutations also result in altered miRNA processing (Suzuki et al. 2009).

The other tumor suppressor genes that show mutations in 5–10 % CRCs are *SMAD2* and *SMAD3*. Both of these proteins are regulated by TGF-β-mediated receptor phosphorylation (ten Dijke and Hill 2004; Harradine and Akhurst 2006) and are involved in nuclear translocation of SMAD4 leading to transcriptional control of the target proteins. High copy amplification of the oncogene *CMYC* is observed in approximately 5–10 % (Leary et al. 2008), and a moderate increase is reported in more than 30 % of CRCs. c-Myc protein is a transcription factor and regulates genes that regulate cell cycle progression, survival and metabolism (Eilers and Eisenman 2008; Ruggero 2009). *CMYC* is the key target gene regulated by activation of β-catenin by APC inactivation. In addition, post-trascriptional changes such as methylation and microRNAs also modulate the expression of many proteins (Babashah and Soleimani 2011; Babashah 2014). Role of miRNAs in colon cancer progression has recently been reviewed by us (Yu et al. 2014).

2 Colorectal Carcinogenesis and Colon Cancer Stem Cells

Despite recent advances in medicine, 30–40 % of patients with CRC show tumor recurrence. Although the reason for this is not fully understood, the presence of chemotherapy resistant cancer stem cells (CSCs), which are distinct from the bulk of the cells in the tumor, is thought to be one of the primary cause for tumor recurrence (Dean et al. 2005). Over the last decade, the cancer stem cell model has become increasingly accepted as an explanation for cancer development, spread and recurrence.

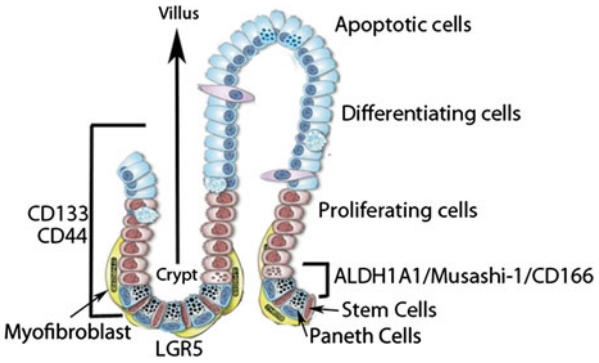

Fig. 9.1 *Schematic diagram showing expression patterns of normal intestinal stem cell andCSC-markers in normal small intestine and colon*. The intestinal epithelium undergoes continous generation and differentiation along the crypt-villus axis. Stem cells residing near the bottom of the crypt give rise to rapidly proliferating progenitor cells, which subsequently differentiate into functional enterocytes (Adapted from Lin et al. Toxins (Basel) 2010)

The CSCs are thought to originate from stem, progenitor or the differentiated cells that have acquired mutations in tumor suppressor genes and/or oncogenes as described above. Like normal stem cells CSCs possess two essential properties: their long-term self-renewal property and their ability to give rise to one or more differentiated cell lineages (pluripotency). In addition, these cells are capable of giving rise to tumor, even when injected in small numbers.

In the normal intestine or colon, stem cells are undifferentiated, multipotent and self-renewable cells that are found towards the bottom of the crypt in the proliferative zone and are also responsible for generating all epithelial cell types along the crypt-villus axis (Fig. 9.1) and maintaining tissue homeostasis and repair. Two types of stem cells have been reported: the LGR5$^+$ crypt base columnar cells (CBCs) and the quiescent DNA label-retaining intestinal stem cells (LRCs) marked by the expression of polycomb group gene Bmi1 (Sangiorgi and Capecchi 2008). Both of these cell types are present in the small intestine, but the presence of LRCs in the colon has not been confirmed. In vitro studies utilizing single LGR5$^+$ CBCs showed organoid formation and crypt domains containing all lineages of the adult intestinal epithelium including enteroendocrine and crypt paneth cells confirming true multipotent nature of these stem cells (Sato et al. 2009). Like LGR5$^+$ CBCs, the Bmi1$^+$LRCs also form spheroids in vitro containing all differentiated epithelial cell types (Yan et al. 2012; Sato et al. 2009). The stem cell characteristics of these cells have been further strengthened by the fact that these cells can restore radiation ablated mouse intestinal epithelium in the total absence of Lgr5$^+$ stem cells (Yan et al. 2012). However, Buczacki et al have identified quiescent LRCs not as the stem cells but rather as partially-differentiated secretory precursors (Buczacki et al. 2013). It is suggested that both these cell types co-exist in the intestinal epithelium, LGR5$^+$ cells comprise the active population of the crypt, whereas Bmi$^+$ cells are quiescent SCs that represent a reserve pool of SCs that replace the LGR5$^+$ cells in case of loss or injury (Medema and Vermeulen 2011; Tian et al. 2011).

Since stem cells proliferate throughout life, they are more susceptible to accumulate oncogenic mutations than differentiated cells with their comparatively short life span. On the other hand, it is also assumed that differentiated cells reacquire stem cell like characteristics by reactivating signaling pathways that are linked to malignant transformation such as the Wnt/β-catenin and Bmi1 pathways or certain Hox genes.

As mentioned above, colorectal cancer results from a series of genetic/epigenetic alterations that transform the normal colonic mucosa into an aberrant phenotype (Markowitz and Bertagnolli 2009; Lampropoulos et al. 2012). The cell that acquires mutation and becomes the first tumor cell is termed the cell of origin. The development of heterogenous tumor from the tumor initiating cell can be explained according to stochastic or CSCs model. The stochastic model suggests that every cell within a tumor is capable of both initiation, propagation and further mutations, whereas CSC model posits that a few cells within the tumor have the potential to propagate the tumor. Increasing evidence is now supporting the latter model or a combination of the two.

Considering that the appearance of CSCs might be one of the initial events in neoplastic transformation in solid tumors as well as in intestinal neoplasia, we investigated the status of CSCs in normal appearing colonic mucosa during aging in patients with adenomatous polyps. Colon CSCs, as evidenced by the expression of CSC markers (CD44, CD166 and Ep-CAM) were observed not only in premalignant adenomatous polyps, but also in normal appearing colonic mucosa, where expression increased with advancing age indicating increased risk of developing colorectal cancer during aging (Patel et al. 2009). Additionally, we found the age-related increase in adenomatous polyps in the colon was associated with increased expression of colon CSC markers (Patel et al. 2009).

3 Identification of Colon Cancer Stem Cells

Identification and isolation of the CSC responsible for tumor initiation and propagation is a huge challenge due to the complexity of their biology and expression of cell surface markers, which differ between tissue types and also other unsolved technical issues. Three methods are usually employed for isolation and characterization of CSCs: (a) isolation based on their drug efflux property by flow cytometric sorting of a side population (SP); (b) colonosphere formation, which are considered as surrogate tumors; (c) sorting on the basis of cell surface markers, which are described in more detail in the following section.

Based on cancer stem cell properties, several investigators identified various membrane and cytoplasmic CRC stem cells markers such as CD133 (O'Brien et al. 2007; Puglisi et al. 2009; Ricci-Vitiani et al. 2007), CD24, CD29, CD44 (Dalerba et al. 2007), CD166 (ALCAM) (Dalerba et al. 2007), EpCAM (ESA) (Ricci-Vitiani et al. 2007), Musashi 1 (Msi-1) (Glazer et al. 2012), Lgr5 (Das et al. 2010), ALDH1 (Todaro et al. 2010). Presence of these proteins has been associated with stem-ness

and generation of tumors reiterating the primary tumor with increased clonogenic ability and multi-lineage potential and has also been associated with tumor stage, differentiation, invasiveness, metastasis formation as well as prognosis (Dick 2008; Reya et al. 2001; Vaiopoulos et al. 2012; Wang and Dick 2005).

CD133 (prominin-1), a transmembrane glycoprotein, was identified as a potential CSC marker for brain tumor (Singh et al. 2004) and several histological variants of tumors, including colon. Later, it was demonstrated that CD133 positive cells from colon metastases formed SCID mice xenografts that resembled the original tumor, whereas CD133-negative cells did not form metastases in mice even when injected in high numbers (O'Brien et al. 2007; Ricci-Vitiani et al. 2007). Both reports point out that the vast majority of CD133 positive cells were not CSC. On the contrary, Shmelelkov et al (Shmelkov et al. 2008) demonstrated that CD133 negative cells had the same tumor initiating capacity as CD133 positive cells and that CD133 was expressed equally in differentiated and undifferentiated cells in the normal human colon. Other investigators also did not observe an enhanced tumor initiating capacity by the presence or absence of CD133 whether the cells were isolated from primary colon tumors or colon cancer cell line (Chu et al. 2009; Feng et al. 2010; Ricci-Vitiani et al. 2007). These differences to form CD133 tumor from primary tumor largely depend on the methodology. However, no functional data are available to date and conflicting results have been reported regarding its role as a true CSC marker (Kemper et al. 2010; Puglisi et al. 2011). In colorectal cancer, CD133 expression is not restricted to rare cell subsets, but it is detectable in a large heterogenous populations of tumor cells, irrespective of their tumorigenicity (Shmelkov et al. 2008). The co-expression on tumor cells of Msi-1, CD44, CD166, and EpCAM molecules has been reported to identify the CSC pool more precisely than CD133 expression alone (Dalerba et al. 2007; Todaro et al. 2008; Vermeulen et al. 2010). Todaro et al. (2010) reported that only tumorigenic $CD133^+$ cells were able to generate colonies organized in crypt-like structure under differentiation conditions on Matrigel. Another study demonstrated that neither over-expression nor loss of CD133 was significantly associated with tumor progression or survival (Langan et al. 2013; Lugli et al. 2010). Muraro et al. (2012) evaluated the correlation of the expression of CD133 or the co-expression of CD166/CD44 or CD24/CD44 with several CSC functional properties but it did not appear to reliably identify CSC populations in established CRC cell lines. Despite the conflicting reports of CRC-SC's to be utilized as clinically relevant biomarkers, CD133 is identified as a potential prognostic marker in a number of cancers (Grosse-Gehling et al. 2013; Ozawa et al. 2014; Pirozzi et al. 2013; Ren et al. 2013; Yamamoto et al. 2014; Yang et al. 2011) and however, little is known about the prognostic value of non-CD133 CRC-SC markers.

CD166 (ALCAM) expression is pathologically correlated with aggressive disease in a variety of cancers and aberrant cell surface CD166 expression is strongly correlated with a shortened survival (Levin et al. 2010; Weichert et al. 2004). It has been reported that loss of membrane CD44, CD166 and EpCAM from normal to early colorectal cancer is linked to tumor progression. This is attributed to loss of their cell adhesion function (Lugli et al. 2010), which is known to be fundamental

Table 9.1 Cancer stem cell marker specific survival in colorectal cancer

Stem cell marker expression	Five year survival rate
High CD133 expression, regardless of CD44 or CD166 expression	44 ± 8.6 % (lowest)
Tumors with high expression of CD44 and/or CD166 regardless of other markers	77 ± 6.1 % (intermediate)
Tumors with low/none expression of all markers	87 ± 9 % (highest)

to initiation of the metastatic process (Woodhouse et al. 1997). A significant increase in CD166 expression in adenomatous glands and an age-dependent increase in CD44 and CD166 expression has been reported suggesting a role for CD44 and CD166 in tumor development from the pre-cancerous state (Patel et al. 2009). Horst et al (Horst et al. 2009) reported colon cancer patients specific survival based on the expression of stem cell markers using the Kaplan-Meier method (Table 9.1).

Msi-1 was also identified as a putative colon SC marker. Most of the Msi-1$^+$ cells were located at the base of human colon, between cell position 1 and 10: a distribution that is believed to maintain the undifferentiated state of SCs (Battelli et al. 2006; Imai et al. 2001; Nakamura et al. 1994; Nishimura et al. 2003). The integrin subunit β1 (CD29) has been reported as a surface marker for the proliferative zone of the human colonic crypt. CD29 is expressed highly in lower third part of the colonic crypt, which harbors stem cells and progenitor cells (Fujimoto et al. 2002).

CD44, a cell surface glycoprotein, first identified as a stem cell marker in breast cancer, is involved in tumor invasiveness, migration and malignant progression to metastases and recently has also been described as a putative colorectal CSC marker (Visvader and Lindeman 2008). More recently, several investigators used lineage-tracking experiments to identify unique markers of normal colon SCs, and identified an orphan G-protein-coupled receptor, Wnt target gene Lrg5 (leucine-rich repeat-containing G protein-coupled receptor 5 (Barker et al. 2007, 2009; Sato et al. 2009; Schepers et al. 2012). There are controversial reports about the role of LRG5 as a tumor suppressor or oncogene in colorectal cancer. Walker et al reported that loss of LRG5 expression increased tumorigenicity and invasion, whereas increased expression of LRG5 inhibited tumorigenicity and clonogenicity (Walker et al. 2011). On the contrary, colorectal cancer patients with high Lrg5 expression were associated with poor prognosis (Han et al. 2015).

Aldehyde dehydrogenase 1 (ALDH1) is a detoxifying enzyme and another potential colon cancer SC marker which is positive with subsets of CD44$^+$ or CD133$^+$ cells and located at the base of the normal crypt. All three markers increased during colon tumor progression to carcinoma (Huang et al. 2009). Increased expression of ALDH1 was associated with poor clinical outcome in colon cancer patients (Goossens-Beumer et al. 2014), whereas Fitzgerald et al. (2014) reported that ALDH1 expression did not increase with progression from normal colon to primary tumors and metastases.

A recent report revealed that high levels of lipid droplets are distinctive marks of CSCs (Tirinato et al. 2014). CRCs exhibited more lipid droplets compared to differentiated tumor or normal epithelial cells (Krahmer et al. 2009; Tirinato et al.

2014). Lipid droplets are dynamic cytosolic lipid strorage organelles. Differential expression of lipid droplets is associated with disease and a possible functional or metabolic link of lipid droplets in CR-CSC is postulated (Bozza and Viola 2010; Farese and Walther 2009). Flow cytometric analysis revealed CD133 expression in lipid droplet containing CRCs and that these CRC-SC retain tumorigenic potential in vivo (Tirinato et al. 2014).

4 Colon Cancer Stem Cells and Metastasis

Tumor recurrence associated with metastasis is by far the biggest clinical challenge associated with cancer. Cancer recurrence and metastasis is dependent on the ability of some cells to detach from the primary location, implant at a separate site and generate secondary tumor. As mentioned earlier, as per the stochastic model, the tumor heterogeneity originates from aberrant mutations within the initial tumor mass as well as due to various micro-environmental influences (Vries et al. 2010; Visvader and Lindeman 2008). Out of the diverse population within the tumor, only selected clones can migrate and form metastasis. On the other hand according to the CSC model, only CSCs can migrate and as a result the metastatic tissue resembles the pattern of original lesion. These cells can undergo further genetic and epigenetic alterations and evolve into new and more malignant CSCs and drive tumor migration and metastasis rather more effectively than the original CSCs (Visvader and Lindeman 2008; Vries et al. 2010). In order for the metastatic process to succeed, a cancer cell should be able to survive under attachment-free conditions, migrate and invade through surrounding stroma, intravasate into the vascular system, survive the rigors of the blood flow, extravasate into an advantageous distant environment, adhere and proliferate. Vast genetic changes including mutations in APC, K-Ras, TP53, PIK3CA, SMAD4 genes and activation of signaling pathways such as Wnt, Notch and Hedgehog, enable these cells to successfully accomplish this intricate process (reviewed in (Rattan et al. 2012)).

Tumor microenvironment plays an important role in helping these stem cells gain tumor-promoting traits (Burness and Sipkins 2010). It has been postulated that a normal intestinal niche can prevent tumor growth even if CSCs are present (Bissell and Labarge 2005). The tumorigenic niche is composed of transformed fibroblasts, recruited myeloid cells, other cell types and extracellular components, which produce many growth factors and cytokines including TGF, HGF, TNF-α, IL-6, EGF and IGF that promote dedifferentiation, carcinogenesis and invasiveness (Medema and Vermeulen 2011; Vermeulen et al. 2010). These extrinsic factors have also been reported convert non-CSCs to CSCs through a process called EMT. In this event simultaneous down-regulation of epithelial phenotype along with enabling of fibroblast-like traits, enhances motility, invasiveness and resistance to apoptosis (Chaffer and Weinberg 2011; Singh and Settleman 2010). Investigations have shown that stimulation of pathways like Wnt, Notch, hypoxia, integrins and PI3K/Akt result in EMT-related changes (reviewed in (Rattan et al. 2012)). Therefore, the

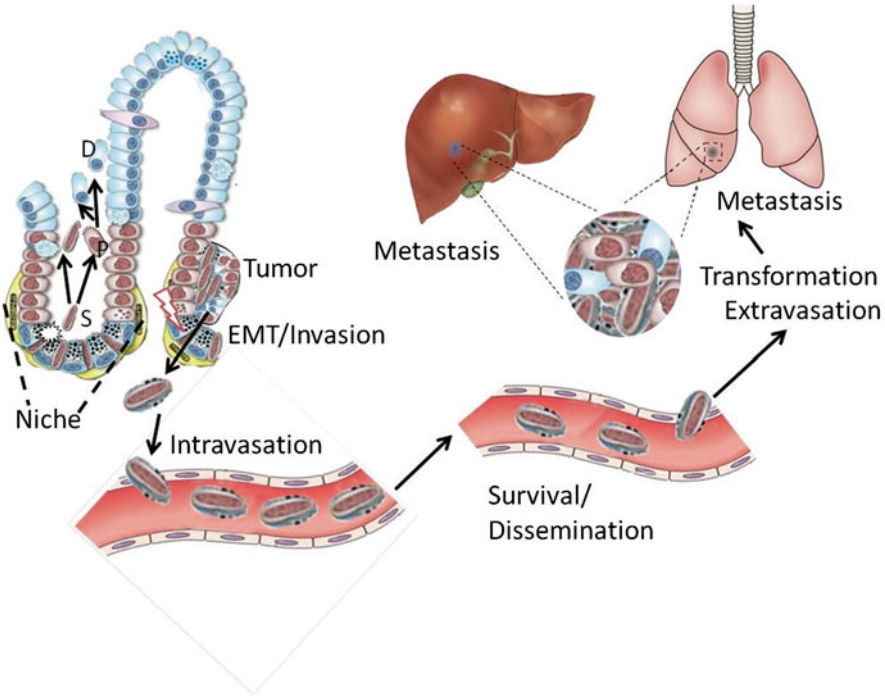

Fig. 9.2 Stem cell model of metastasis. Stem cells (S) give rise to progenitor (P) and differentiated (D) cells in the normal course of events. The CSCs that have undergone EMT due to genetic and epigenetic mutations (lightening bolt) are released from the primary tumor into the blood vessels. The microenvironment or the niche supports this transition. The invaded cells travel to distant locations, where they undergo transformation and form metastasis

circulating tumor cells are expected to have stem-like characteristics. Indeed, presence of stemness markers in the peripheral blood of cancer patients is associated with worse prognosis and recurrence (Iinuma et al. 2011; Gazzaniga et al. 2010). A stem cell model of colon cancer metastasis is shown in Fig. 9.2.

5 Chemoresistance in Colon Cancer Stem Cells

5.1 CSCs Are Self-Renewing Cells with a Low Proliferation Rate

A low rate of multiplication is a hallmark of the somatic stem cells of normal tissues. The presence of quiescent cells with CSC properties has been demonstrated in several tumor systems, using retention of DNA label or lipophilic dye. CSCs can divide to yield a more differentiated cell and a daughter cell that maintains the same properties as the parental cell. This ability of self-renewal in CSCs drives tumor

growth, metastasis and recurrence. Actually, despite their capacity for self-renewal, CSCs are relatively quiescent; that is, they have low proliferative rate and are often not cycling. Indeed, they have been shown to have significantly longer cell cycle times than proliferating non-stem cells. We have reported that the growth of CSCs from colonospheres formed by colon cancer HCT-116 and HT-29 cells remains 30–40 % lower than the corresponding parental cells (non-CSCs) (Kanwar et al. 2010). This is presumably due to the arrest of CSCs at a G0-like cell cycle phase or checkpoint (Paldino et al. 2014). Touil and colleges have recently reported that 5-FU resistant colon cancer cell population expresses a typical cancer stem cell-like phenotype and enter into a reversible quiescent G0 state upon re-exposure to 5-FU (Touil et al. 2014). Quiescent CSC avoid DNA damage induced by chemotherapeutic drugs, because these agents primarily hit cells in the S-phase cycle. Hence, cells in the G0/G1 phase of the cell cycle are thought to be relatively resistant to classical cytotoxic therapy (Stewart et al. 2007). Yan and colleges have reported that Bmi1 and Lgr5 mark two functionally distinct crypt intestinal stem cells (ISCs) in mice. $Lgr5^+$ ISCs are mitotically active ISCs, Bmi1 marks quiescent ISCs that are insensitive to Wnt perturbations, contribute weakly to homeostatic regeneration, and are resistant to high-dose radiation injury. Clonogenic culture of isolated single $Bmi1^+$ ISCs yields long-lived self-renewing spheroids of intestinal epithelium that produce Lgr5-expressing cells, thereby establishing a lineage relationship between these two populations in vitro (Yan et al. 2012). In conclusion, quiescent CSC have enough time to avoid, to reduce and to repair drugs induced DNA damage or cytotoxicity.

5.2 Side-Population and ABC Transporters in CSC Cells

Side population (SP) cells can rapidly efflux lipophilic fluorescent dyes to produce a characteristic profile based on fluorescence-activated flow cytometric analysis. Previous studies demonstrated SP cells in bone marrow from patients with acute myeloid leukemia, suggesting that these cells might be candidate leukemic stem cells. Recent studies have found that many types of cell lines and tissues including colon cancer cell lines to contain SP cells. Studies on testicular stem cells indicate that more than 40 % of the SP (defined in this case as cells that show higher efflux of DNA-binding dye Hoechst 33342) were undifferentiated spermatogonia, while other differentiated fractions were represented by only 0.2 % (Takubo et al. 2008). We have observed 80 % increase in dye exclusion in CSCs which were derived from colonospheres of HCT-116 cells (Kanwar et al. 2010). This was associated with increased expression of ABC transporter protein ABCG2, a member of the superfamily of ATP-binding cassette (ABC) transporters whose primary function is to transport various molecules across the intra- and extra-cellular membranes (Fletcher et al. 2010).

ABCG2, also termed BCRP/MXR/ABCP, was independently cloned from placenta as well as cell lines selected for resistance to mitoxantrone or anthracyclines. An increased expression was also observed in the human colon CSC that were

enriched in chemo-residual and chemoresistant colon cancer cells (Kanwar et al. 2010; Yu et al. 2009, 2013). ABCG2 consists of a nucleotide-binding domain (NBD) at the amino terminus and a transmembrane domain (TMD) at the carboxyl terminus and it is postulated to form a homodimer to perform its biological functions. Over-expression of ABCG2 in cell lines confers resistance to a wide variety of anticancer drugs including mitoxantrone, daunorubicin, doxorubicin, topotecan and epirubicin. The expression of ABCG2 has been implicated in multidrug resistance (MDR) of acute myeloid leukemia and some solid tumors. In addition, ABCG2 can transport several fluorescent dyes or toxins. ABCG2 is found to be expressed in epithelial cells of intestine and colon, liver canaliculi, and renal tubules, where it serves to eliminate the plasma level of orally administered anticancer drugs as well as ingested toxins.

Recently, Xiong et al. (2014) reported isolation of SP cells by fluorescence-activated cell sorting (FACS) from multiple human colon cancer cell lines. Each cell line contains only about 1 % SP cells. These SP cells could differentiate into SP and non-SP cells. SP cells had a higher proliferation potency than non-SP cells. Compared to non-SP, SP cells showed increased mRNA and protein expression of drug export transporters (ABCG2, MDR1), stem cell growth related pluripotency factors (OCT-4, NANOG, SOX-2) and CSC marker (CD44, CD133). Moreover, SP cells were more resistant to chemotherapeutic drug 5-FU and cisplatin and were more invasive and displayed increased tumorigenic ability than their non-SP counterparts. They also exhibit higher expression of drug export transporters such as ABCG2 and lead therapeutic resistance in colon cancer.

5.3 Epithelial to Mesenchymal Transition

There are two major forms of drug resistance: *de novo* or acquired. Patients who are initially refractory to therapy display intrinsic or "de novo" drug resistance. Patients that initially respond to therapy typically relapse as a consequence of "acquired" drug resistance (Singh and Settleman 2010).

Recent studies have reported that CSCs or CSC-like cells are enriched in tumor remnants after chemotherapy. These include glioma, breast cancer, colon cancer and sophisticated CML mouse model (Clevers 2011). Data from our laboratory have demonstrated that although the combination of 5-FU and Oxaliplatin (FUOX) inhibited the growth of human colon cancer HCT-116 or HT-29 cells growth, it led to enrichment of CSC phenotype (Yu et al. 2009). We have now generated FUOX-resistant HCT116 and HT29 cells that exhibit both enrichment of CSCs/CSLCs and elevated levels of microRNA-21 (miR-21). Further, we have demonstrated that miR-21 plays a determinant role in inducing stemness in colon cancer cells (Yu et al. 2009, 2012).

Although the precise mechanism of acquired resistance is unclear, it is suggested that differentiated or undifferentiated cancer cells or CSCs adjust their gene expression profile, which is regulated by CSC niche. A simple example is that the cancer

cells increase their expression of thymidylate synthase (TS) after 5-FU treatment (Peters et al. 2002). A massive adjusting or reprogramming of gene expression leads to epithelial to mesenchymal transition (EMT).

Conventionally, EMT are trans-differentiation programs that are required for tissue morphogenesis during embryonic development. Recent reports indicate that the emergence of CSCs occurs in part as a result of EMT and EMT of tumor cells not only causes increased metastasis, but also contributes to drug resistance. The relationship of EMT, CSC and drug resistance have been summarized by Singh and colleagues (Singh and Settleman 2010).

6 Development of Cancer Stem Cell Targeted Therapies

6.1 CSC Targeted Therapy

One of the characteristics of CSCs is the expression of a distinctive set of surface biomarkers, which has led to the identification of key cellular activities that makes them vulnerable to therapeutic interventions. For instance, cell surface marker expression could be used for antibody-directed therapy to target proteins such as CD133, CD44 or EpCAM (Deonarain et al. 2009). ABCG2, which are ATP-dependent drug efflux pumps, could be targeted by ATP-competitive agents (Kuhnle et al. 2009; Robey et al. 2007). CSC- targeted therapy should be combined with conventional therapeutic agents that can eliminate both differentiated and undifferentiated cancer cells in order to avoid recurrence of tumor due to dedifferentiation of cancer cells into CSCs by the process of EMT.

6.2 Induction of CSCDifferentiation and Treatment

While CSCs are resistant to conventional chemotherapy, differentiated or differentiating cells that form bulk of the tumor, are sensitive to chemotherapy. Therefore, the differentiation induction is one of the therapeutic options proposed to eliminate or functionally antagonize CSCs. This therapeutic strategy consists of forcing CSCs to shift into a terminal epithelial phenotype, losing their self-renewal abilities, and therefore becoming vulnerable to conventional therapies (Paldino et al. 2014).

Recently, few molecular agents including bone morphogenetic protein 4 (BMP4), antisense oligonucleotides (anti-miR-21) and some natural compounds like difluorinated curcumin (CDF) and Omega-3 polyunsaturated fatty acids (ω-3 PUFA) have been proposed to induce differentiation in colon CSCs. BMP4 is able to activate a differentiation program and stimulate apoptosis in colon CSCs, reducing β-catenin activation through inhibition of PI3K/AKT pathway and up-regulation of Wnt-negative regulators. Additional, administration of BMP4 to immune-compromised mice with tumors, which arose from colon CSCs, increased the antitumor effects of

5-fluorouracil and oxaliplatin, confirming that BMP4 might be developed as a therapeutic agent against cancer stem cells in advanced colorectal tumors (Kanwar et al. 2011; Paldino et al. 2014).

MicroRNAs (miRNAs, miRs) are endogenous posttranscriptional modulators that negatively control the expression of their target genes and play an important role in the development and progression of many malignancies, including colorectal carcinoma. In particular, expression of miR-21 is greatly increased in chemotherapy-resistant colon cancer cells that are enriched in undifferentiated CSCs (Yu et al. 2009, 2012). Down-regulation of miR-21 in chemoresistant colon cancer cells by antisense miR-21 induced differentiation, as evidenced by marked increases in cytokeratin-20 (CK-20) expression and alkaline phosphatase activity (Yu et al. 2013). These changes were accompanied by a significant reduction in the expression of colon CSC marker CD44, colonosphere formation, and T-cell factor/lymphoid enhancer factor (TCF/LEF) activity but increased the expression of proapoptotic programmed cell death 4 gene (Yu et al. 2012). Induction of differentiation greatly increased sensitivity of chemoresistant colon cancer cells to the chemotherapeutic agents 5-FU, oxaliplatin and the combination of 5-FU and oxaliplatin (FUOX) (Yu et al. 2013).

Treatment of CSC-enriched chemoresistant colon cancer cells with CDF $^{+}$FUOX showed a higher magnitude growth inhibition by either agent alone. Growth inhibition by CDF and CDF^{+} FUOX in differentiating CR colon cancer cells was associated with reduction in the expression of CD44 and epidermal growth factor receptor (EGFR) (Yu et al. 2013; Kanwar et al. 2011). The observation suggests that down-regulation of miR-21 induces differentiation of CSCs and differentiation enhances susceptibility of CR cancer cells to conventional and nonconventional therapeutic regimen.

More recently, we reported that eicosapentaenoic acid (EPA; one of the ω-3 PUFA) alone was effective, combination of EPA and FUOX was more potent in inhibiting the growth of CSC- enriched chemoresistant colon cancer cells as evidenced by decreased colonosphere formation and increased sphere disintegration as well as suppression of growth of xenografts of CR colon cancer cells in SCID mice, and lastly reduction in proinflammatory metabolites in mice (Vasudevan et al. 2014). In addition, EPA^{+} FUOX increase apoptosis as evidenced by PARP cleavage and resulting reduction in CSC/CSLC population. Furthermore, increased pPTEN, decreased pAkt, normalization of β-catenin expression, localization, and transcriptional activity were observed by EPA^{+} FUOX treatment (Vasudevan et al. 2014). The data suggest multiple signaling pathways are involved in regulation of self-renewal of CSCs.

7 Future Directions

The drugs under development mainly attempt to target signaling pathways involved in the regulation of self-renewal of normal somatic stem cells, such as the Wnt, the Sonic Hedgehog and the Notch pathways. The focus needs to be shifted towards the

development of drugs that would either preferentially block stem cell (and CSC) renewal or drive the stem cells into differentiation, thus closing down the tumor supply line (Zhou et al. 2009; Frank et al. 2010). A thorough understanding of stem cell biology in terms of signaling and proliferation is essential for the development of therapeutic strategies to eliminate them. A major hurdle in achieving a successful therapeutic modality is specificity. Small molecules or chemically modified oligonucleotides such as anti-miR (Stenvang et al. 2012) that target multiple pathways involved in stem cell self-renewal provide an excellent therapeutic modality (Kreso et al. 2014).

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Chapter 10
Pancreatic Cancer Stem Cells

Deepak Ray, Reyhaneh Khoshchehreh, Alexandra Aicher, and Christopher Heeschen

Abstract Various levels of evidence suggest that a small population of tumor cells known as cancer stem cells (CSCs) or tumor initiating stem like cells initiate and maintain tumors. CSCs have been identified in pancreatic cancer ductal adenocarcinomas (PDAC) and are known to self-renew and propagate the parental tumor. The lack of early symptoms, extensive metastasis and high resistance to chemotherapy and radiation render pancreatic cancer the fourth most common cause of cancer related death. Tumor initiating/propagating cells express cell surface markers such as CD133 and CD44 and show features of epithelial-mesenchymal transition (EMT) resulting in metastasis. In addition, densely glycosylated proteins known as mucins are found to be associated with pancreatic CSCs and play a role in EMT. Typically, activating mutations in the *Kras2* gene are detected in pancreatic tumors accompanied by inactivating mutations in tumor suppressor genes such as *Arf* or *P53*. The cell-of-origin of PDAC is still unknown, as both exocrine and endocrine cells can initiate tumors during chronic inflammation. Future studies investigating pancreatic stem cells and progenitor cells in more detail will help identify more precisely the cell-of-origin of PDAC. Understanding the underlying molecular pathways of the metastatic and drug resistant nature of these distinct cells will open up new avenues in targeting these cells. The highly heterogeneous pancreatic CSC pool is more resistant to standard chemotherapy than the more differentiated tumor cells, and therefore, strategies to specifically target PDAC CSCs will provide new therapeutic prospects for this devastating disease.

Keywords Pancreatic adenocarcinoma • Pancreatic progenitors • Pancreatic cancer stem cells • Metastasis-initiating cells • Chronic inflammation • Immunotherapy

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1 Pancreatic Cancer Biology and Pathology

1.1 The Normal Pancreas

The pancreas is an abdominal organ about 6 in. long and less than 2 in. wide in adults. The head of the pancreas is on the right side of the abdomen, posterior to the junction between the stomach and the duodenum. The body of the pancreas is located posterior to the stomach, and the tail of the pancreas is on the left side of the abdomen adjacent to the spleen. The pancreas contains both exocrine and endocrine glands. The exocrine pancreas contains acinar cells that produce zymogens for food digestion. Following activation of these zymogens, other digestive enzymes such as trypsin, amylase or carboxipeptidase are activated and reach the small bowel. Ductal cells produce digestive juices that are transported to the gut, while also secreting mucins and bicarbonate to raise the duodenal pH. More than 95 % of the cells in the pancreas are contained in the exocrine glands and ducts. The endocrine pancreas also produces insulin, glucagon, somatostatin, ghrelin and P peptide in the islets of Langerhans.

1.2 Pancreatic Cancer

In contrast to the decreasing mortality for other tumor types resulting from improved prevention and treatment options, pancreatic cancer shows a growing incidence and prevalence in defiance of increasing preclinical and clinical efforts (Ryan et al. 2014) (Cancer Facts & Figs. 2011. American Cancer Society, http://cancer.org). Pancreatic cancer has an overall 5-year survival rate of around 5 % and is the fourth most frequent cause for cancer-related deaths, (Hidalgo 2010). Only lung, colorectal and prostate cancers have a higher incidence. The disease has a poor prognosis due to a lack of early warning signs and typically presents with extensive metastasis upon initial diagnosis. Moreover, pancreatic cancer is highly resistant to conventional chemotherapy and radiotherapy. Surgery is generally not curative as there is generally extensive spread of the disease at presentation, and in some cases surgery is precluded by tumor invasion of adjacent large vessels.

Worldwide, the incidence of all types of pancreatic cancer (85 % of which are adenocarcinomas) ranges from 1 to 10 cases per 100,000 people, is generally higher in developed countries and among men, and has remained stable for the past 30 years relative to the incidence of other common solid tumors (Jemal et al. 2011). PDAC is rarely diagnosed in persons younger than 40 years of age, and the median age at diagnosis is 71 years. It is the eighth leading cause of death from cancer in men and the ninth leading cause of death from cancer in women throughout the world. In the United States, pancreatic cancer is expected to develop in 46,000 people and 40,000 people are expected to die from it per year (Siegel et al. 2014). Discrimination between exocrine and endocrine cancers of the pancreas is crucial,

as the different cell types of the pancreas form different types of tumors. The diverse pancreatic cancers have distinct risk factors and causes, different warning signs and symptoms, are diagnosed using different tests, have different treatment regimens, and show different prognosis.

1.3 Exocrine Tumors

Exocrine tumors are by far the most common type of pancreas cancer including:

– Pancreatic ductal adenocarcinoma (PDAC): These cancers usually begin in the ducts of the pancreas.
– Solid pseudopapillary neoplasms (SPNs): These are rare, slow-growing tumors that almost always occur in young women.
– Ampullary cancer (carcinoma of the ampulla of Vater): This cancer starts in the ampulla of Vater, which is where the bile duct and pancreatic duct come together and empty into the small intestine.
– Less common types of cancers: Other cancers of the exocrine pancreas include adenosquamous carcinomas, squamous cell carcinomas, signet ring cell carcinomas, undifferentiated carcinomas, and undifferentiated carcinomas with giant cells.

1.4 Endocrine Tumors

Tumors of the endocrine pancreas are uncommon, making up less than 4 % of all pancreatic cancers. As a group, they are sometimes known as *pancreatic neuroendocrine tumors (NETs)* or *islet cell tumors*. There are many types of pancreatic NETs:

– Functioning tumors: About half of pancreatic NETs make hormones that are released into the blood and cause symptoms. These are called *functioning* tumors and each one is named for the type of hormone-making cell it starts in.

- Gastrinomas come from cells that make gastrin. About half of gastrinomas are cancers.
- Insulinomas come from cells that make insulin. Most insulinomas are benign.
- Glucagonomas come from cells that make glucagon. Most glucagonomas are cancers.
- Somatostatinomas come from cells that make somatostatin. Most somatostatinomas are cancers.
- VIPomas come from cells that make vasoactive intestinal peptide (VIP). Most VIPomas are cancers.
- PPomas come from cells that make pancreatic polypeptide. Most PPomas are cancers.

The most common types of functioning NETs are gastrinomas and insulinomas. The other types occur very rarely.

– Non-functioning tumors: These tumors do not make enough excess hormones to cause symptoms. They are more likely to be cancer than functioning tumors. Because they do not make excess hormones that cause symptoms, they can often grow quite large before they are found.

1.5 Risk Factors and Biologic Features of PDAC

PDAC arising from the exocrine pancreas is the most common and lethal of these various pancreatic cancers, and causes for its development remain unknown. Several environmental factors have been implicated, but evidence of a causative role exists only for tobacco use. The risk of pancreatic cancer in smokers is 2.5–3.6 times higher than in non-smokers; the risk increases with greater tobacco use and longer exposure to smoke. The specific carcinogens in tobacco smoke are not well characterized, but cadmium is a likely candidate (Amaral et al. 2012). Interestingly, nicotine, although non-carcinogenic, accelerates *Kras*-initiated pancreatic cancer development and progression by altering the acinar cell compartment (i.e. dedifferentiation) and making it more susceptible to oncogenic transformation (Hermann et al. 2014).

For non-smokers, food intake is the major source of cadmium, which may derive from fertilizers or fossil fuel combustion (Luckett et al. 2012). Data are limited on the possible role of alcohol intake as a contributing factor. Some studies have shown an increased incidence of pancreatic cancer among patients with a history of diabetes or chronic pancreatitis. There is also evidence, though less conclusive, that chronic cirrhosis, a high-fat, high-cholesterol diet, and previous cholecystectomy are associated with an increased incidence of PDAC (Rebours et al. 2015; Sellam et al. 2015). More recently, an increased risk has been observed among patients with blood type A, B, or AB as compared with blood type O (Sun et al. 2015). According to recent studies, regular use of aspirin at a low dose may lower the risk of PDAC (Anderson et al. 2002; Streicher et al. 2014).

Although an estimated 5–10 % of pancreatic cancers have an inherited component, the genetic basis for familial aggregation has not been identified in most cases. A known family history of pancreatic cancer in a first-degree relative is associated with an increased risk of PDAC as compared with the general population, the relative risk being increased by a factor of 2, 6, and 30 in people with one, two, and three affected family members respectively. There is no effective screening tool to detect asymptomatic premalignant or early malignant tumors. Although there is consensus regarding the value of screening patients with an inherited predisposition for pancreatic cancer, there is no consensus on the most effective method of screening or the optimal interval between screenings.

Development of the disease involves an initial pre-invasive state termed pancreatic intraepithelial neoplasia (PanIN) classified into three stages based on increasing cellular atypia and mutations in key oncogenes and specific tumor suppressor genes. 90 % of all PDAC tumors contain mutations in the *KRas2* gene that result in permanently active Ras protein. Ras is a small GTPase involved in proliferation, survival, and differentiation, and is considered a master regulator of PDAC initiation and progression. In mouse models, PanINs and PDAC can be induced by activating mutations in the *Kras* gene (Hingorani et al. 2003; Morris et al. 2010). Other frequent genetic alterations in PDAC are inactivating mutations of *p16* (>95 % of cases) and *TP53* (>50 % of cases). Inactivating mutations of the tumor suppressors *SMAD4* and *BRCA2* are also frequently found in PDAC (Perez-Mancera et al. 2012). Reactivation of developmental embryonic signaling pathways such as Hedgehog and Notch suggests that tumor cells show regression to a dedifferentiated/progenitor-like state and may represent stem/precursor cells.

By far the most common cause of chronic pancreatitis is alcohol abuse, which is responsible for 60–90 % of cases. As with hereditary pancreatitis, the chronic inflammation seen in chronic pancreatitis is thought to predispose to development of PDAC. Inflammatory cytokines may induce cellular proliferation, reduce immunosurveillance and inhibit senescence, all of which enable the lesion to progress to PDAC. The organ-specific microbiome might be of special interest. Although the pancreas does not contain a known microbiome, the organ may be exposed to microorganism-associated molecular patterns and bacterial metabolites via anatomical links with the gut. Lipopolysaccharide derived from bacterial cell walls has been reported to increase pancreatic cancer development (Schwabe and Jobin 2013). Various animal models demonstrate that a germ-free environment or antibacterial treatment reduce tumor incidence.

Chronic pancreatitis may induce an increased level of plasticity in different pancreatic cell types, which favors malignant transformation. In addition, pancreatitis enhances proliferation and inflammatory response in the tissue associated with re-expression of *Pdx1* and reactivation of the embryonic Notch and Hedgehog signaling pathways. Pancreatitis-induced PanINs can be delayed by non-steroidal anti-inflammatory drugs (Guerra et al. 2011). Further study of the underlying mechanisms of chronic inflammation will provide more insights in understanding the link between the various genetic alterations in PDAC and malignant transformation. It is possible that *Kras* and *Trp53* might be mutated in pancreatic stem cells, which then become activated and proliferate due to secretion of inflammation-induced cytokines and growth factors. Therefore, genetically engineered mouse models utilizing specific promoters for tissue stem cells are required to obtain in-depth insights into tumor development and progression. The disease models also need to be investigated in the presence or absence of microorganism-associated molecular pattern and bacterial metabolites.

2 Pancreatic Stem Cells and Their Role in Pancreas Development

2.1 Pancreas Development and Precursors

During the embryonic developmental stage, the pancreas develops as dorsal and ventral evaginations from the foregut endoderm during the fifth week of gestation. Cells from the dorsal and ventral buds slowly undergo lineage commitment to the either endocrine or exocrine compartment. The endocrine compartment comprises the islets of Langerhans while the exocrine compartment is organized into acinar, ductal and centroacinar cells. In addition to these compartments, a novel gland like mucinous compartment known as the pancreatic ductal gland has been identified, and shown to possess a characteristic molecular signature. Much effort has been expended in efforts to identify which of these compartments give rise to PDAC progenitor cells.

The dorsal and ventral buds of embryonic pancreas are organized in a stratified epithelium comprising early multipotent pancreatic cells and early-differentiated endocrine cells, in particular glucagon$^+$ cells. At an early stage, these multipotent pancreatic cells still show a high level of plasticity and can be reprogrammed to an intestinal lineage. In contrast, at later stages of development, multipotent pancreatic cells become committed to the pancreatic lineage and express transcriptional factors for the pancreatic differentiation program (Pan and Wright 2011).

Extensive efforts have been made to identify pancreatic stem cells, which could be involved in the maintenance and/or regeneration of the pancreas in response to injury (e.g. chronic pancreatitis) and loss of β-cell mass, respectively. The characterization of such an elusive stem cell population could lead to the development of therapeutic strategies for the replacement of β-cells in patients with type I diabetes. Despite lacking a clear definition of postnatal pancreas stem cells for the different cell types within the pancreas, comprehensive knowledge has been accumulated regarding the characteristics of pancreatic stem cells during embryonic development. Thus, all pancreatic cells, both from exocrine and endocrine lineages, are believed to originate from an initial cell progenitor expressing the transcription factor pancreatic and duodenal homeobox 1 (Pdx1) (Ahlgren et al. 1996).

The expression of this factor together with silencing of signaling mediated by Sonic hedgehog (Shh) in the surrounding mesenchymal tissue initiates embryonic pancreas development (Apelqvist et al. 1997). The implication of Shh in this process is supported by several observations, including a lack of Pdx1 expression in embryos with constitutively active hedgehog signaling (Hebrok et al. 1998). Thus, Pdx1 can be considered a critical transcription factor in pancreatic commitment, although there might be more actors implicated, since absence of this factor does not result in complete impairment of pancreas formation.

Another transcription factor was recently shown to play an important role in pancreas development in humans. Malfunctioning mutations of pancreas-specific transcription factor 1 (Ptf1) in humans result in impaired pancreas development,

while studies in mice showed that forced expression of Ptf1 induces pancreas development at ectopic locations. Ptf1 is activated in a subset of pancreatic stem cells expressing Pdx1, shortly after these cells acquired Pdx1 expression, but despite the apparent temporal sequence, the expression of Pdx1 and Ptf1 occurs in an independent manner. Ptf1 expression has been implicated in the commitment of precursor cells towards an exocrine phenotype because Ptf1 null mutant mice show impaired pancreas development but are still capable of developing endocrine cells (Krapp et al. 1998). In addition, commitment towards an exocrine fate seems to be potentiated through signaling of the surrounding mesenchyme on Pdx1 positive cells. Mesenchymal cells would enhance Notch signaling in progenitor cells via its downstream target hairy enhancer of split 1 (Hes1) and inhibit the expression of the pro-endocrine differentiation factor Neurogenin 3 (Ngn3) (Lee et al. 2001).

Determination of endocrine fate is induced by expression of the transcription factor Ngn3. In fact, Ngn3-positive cells represent the origin of all the heterogeneity of pancreatic endocrine cells. Both α- and β-cells can derive from Ngn3-positive cells, although they are generated at different ratios. In early pancreatic development during mouse embryogenesis, the vast majority of cells derived from Ngn3-positive cells are glucagon secreting α-cells, supporting a notion that Pdx1-Ngn3 forced expression primarily leads to the development of glucagon cells. The α-cells down-regulate Pdx1 expression and progress towards a non-epithelial phenotype through a process that strongly resembles Epithelial-to-Mesenchymal Transition (EMT).

On the other hand, β-cells retain Pdx1 expression and remain in low numbers as compared to glucagon secreting cells, until later in development when branching morphogenesis and acinar cell differentiation occur and require an amplification of the pool of β-cells. Commitment towards α- or β-cell fate seems to depend on the mutually exclusive action of the transcription factors, Aristalless related homeobox (Arx) and paired box gene 4 (Pax4) (Collombat et al. 2005; Collombat et al. 2003). Expression of Arx may induce the formation of α-cells, since deletion of this gene results in impaired generation of this cell type, whereas Pax4 appears to be responsible for β-cell formation.

The existence of different sequential progenitor cells raises the question of whether these cells can also be reverted to a less differentiated phenotype in order to give rise to a broader number of cell types. However, accumulating evidence suggests that β- cells are differentiated cells with very limited expansion capability. In fact, most β-cells seem to originate from a pool of already existing β-cell precursors rather than from expansion of ancient β-cells. Notch is not capable of compelling mature endocrine cells to revert towards a progenitor-like state. In contrast, Ngn3-positive cells demonstrate greater plasticity, since they can be reverted to a ductal progenitor phenotype. Therefore, while the pancreas lacks a clear hierarchical organization and a final definition of a putative pancreatic stem cell is still missing, it has been shown that a number of cellular compartments bear the potential to regenerate the different subsets of the pancreas and are putative targets for the cell-of-origin for PDAC.

2.2 Multipotent Stem Cells in Neonatal Pancreas

At the time of birth, there are still multipotent pancreatic stem cells, but multipotency is drastically decreased in adult cells. The hepatocyte growth factor receptor c-met identifies cells exhibiting colony-forming activity, while being negative for vascular markers. These c-met$^+$ cells can differentiate into the acinar, ductal and endocrine lineage in vitro and in vivo (Marsit et al. 2004). However, c-met$^+$ cells from adult pancreas lost this multi-lineage potential. Moreover, endocrine-committed CD133$^+$ and CD49f$^+$ pancreatic islet progenitors have been isolated from mouse fetal pancreas that are highly enriched for Ngn3, a consensus marker for progenitors (Sugiyama et al. 2007).

2.3 Multipotent Stem Cells in Adult Pancreas

As shown in other epithelial tissues, tissue resident stem cells with sphere forming capacity have been identified in adult murine pancreas that differentiate into pancreatic exocrine and endocrine lineages, but also into stellate cells and neuronal lineages (Seaberg et al. 2004). In addition, pancreas-derived insulin$^+$ multipotent precursors were isolated from Pdx1$^+$ progenitors in the islets. They are able to differentiate in vivo into β cells, other endocrine cell types, acinar cells, and neural cell types (Smukler et al. 2011). Interestingly, multipotent precursors can be also found in human pancreas suggesting that these multipotent precursors are quite conserved among species. Markers for a direct identification of these multipotent precursors would be helpful. So far, they have been isolated according to their functional characteristics.

Alternative sources of stem cells in the adult murine tissue are centroacinar cells (CACs) and terminal duct cells isolated based on their enhanced ALDH1 activity, increased stemness-associated genes, low levels of pancreatic differentiation markers and Pdx-1, and anchorage-independent cell growth in spheres (Rovira et al. 2010).

3 Cell of Origin of Pancreatic Ductal Adenocarcinoma

The specific cell type from which PDAC arises still remains elusive. A possible scenario for tumor initiation in solid organs is the malignant transformation of stem cells resident in normal tissue. Somatic stem cells are intrinsically endowed with the capacity of self-renewal and would therefore only need to accumulate sequential mutations to undergo malignant transformation and give rise to a tumor. Indeed, this hypothesis has just recently been validated for intestinal cancer. However, putative pancreatic stem cells in mice still cannot be genetically tracked due to their rather

vague description as mentioned above. This has hampered the field in providing definitive proof for this hypothesis. Until this stem cell model for the development of PDAC is either authenticated or disproved by the accumulation of more evidence, other models will need to be considered for a putative mechanism.

The generation of mouse models that closely recapitulate human disease has provided a unique platform for better understanding the cell types that are most susceptible for malignant transformation and may be candidates for the cell-of-origin for murine PDAC (Pérez-Mancera et al. 2012). Depending on the context, mature cells or common multipotent stem cells can undergo initial malignant transformation.

3.1 *Pdx1 Expressing Cells*

The KPC mouse (*Pdx1*Cre; LSL-*Kras*^{G12D}; LSL-*Trp53*^{R172H}) is one of the most frequently used pre-clinical models for human pancreatic cancer. To activate KrasG12D and produce the inactive mutant P53R172H in this model, expression of Cre recombinase occurs in Pdx1^{+} pancreatic progenitor cells during embryonic development resulting in removal of the stop cassette (LSL), and leading to metastatic and chemotherapy-resistant adenocarcinomas with features similar to human disease (Hingorani et al. 2005; Olive et al. 2009). However, this model also has some drawbacks, because the mutant alleles are induced from embryonic day 8.5 when pdx-1 is first expressed in multipotent pancreatic cells in the developing embryo. Moreover, the mutant alleles are activated in both exocrine and endocrine cells, including differentiated cells as well as local resident stem cells, which causes multiple lesions dissimilar to human tumors. Activation of *Kras*^{G12D} alone in Pdx1^{+} cell during embryonic development leads only to premalignant PanINs lesions and not to PDAC. Although theoretically all pancreatic cells can carry the transgene only a few lesions appear, indicating that only a small percentage of Pdx1^{+} cells are targeted.

An inducible *Pdx1*CreER^{T2} has been designed in which the mutant alleles can be switched on during adult life. Activation of *Kras*^{G12D} during adulthood in this model leads to PanIN lesions and acinar-ductal metaplasia. More specific promoters and temporally controlled Cre recombinase have recently been developed, and highlight diverse putative cell types as tumor initiating cells causing PanIN lesions or invasive PDAC.

3.2 *Ductal Cells*

Glandular ductal structures and expression of cytokeratin 19 (CK19) ductal gene are common features of invasive PDAC, and point to cells with ductal phenotype as cell-of-origin in pancreatic cancer. Other lines of evidence however appear to negate this; for instance, expression of *Kras*^{V12G} under the *CK19* promoter induces

inflammation, but not hyperplasia in the pancreas (Brembeck et al. 2003), while activation of *Kras*^{G12D} by CK19-CreERT2 induce PanIN lesions but not adenocarcinomas (Ray et al. 2011). More recent studies conclusively revealed that ductal and stem-like centroacinar cells were surprisingly refractory to oncogenic transformation, whereas acinar cells readily formed PDAC precursor lesions with ductal features (Kopp et al. 2012). It was shown that formation of acinar-derived premalignant lesions depends on ectopic induction of the ductal gene Sox9. Moreover, when concomitantly expressed with oncogenic *Kras*, *Sox9* accelerated formation of premalignant lesions. Although counterintuitive, these results suggest that its precursors arise via induction of a duct-like state in acinar cells.

3.3 Acinar and Centroacinar Cells

As suggested by above studies, acinar cells (including centroacinar cells) are more promising candidates as putative tumor-initiating cells of PDAC. In line with this hypothesis, ductal metaplasia inside acini together with PanIN lesions is a common event following *Kras*^{G12D} activation. If specific promoters for acinar cells are used such as elastase or proCPA1, Expression of mutated *Kras* alleles in combination with mutations in *Trp53* or *Arf* using specific promoters for acinar cells (such as *elastase* or *proCPA1*) lead to induction of PDAC in the presence of cerulean, an inducer of chronic pancreatitis (Gidekel Friedlander et al. 2009; Guerra et al. 2007).

3.4 β Cells

Adult β cells are refractory to transformation by *Kras*^{G12D} alone or in combination with additional mutations. However, in a context of chronic pancreatitis, activation of mutant *Kras* and elimination of tumor suppressor *p53* in insulin^{+} cells by treating *RipCreER*^{TM}; LSL-*Kras*^{G12D}; *Trp53*$^{flox/flox}$ with tamoxifen promote the development of poorly differentiated and undifferentiated adenocarcinomas (Gidekel Friedlander et al. 2009). These adenocarcinomas display a metastatic behavior similar to human tumors. Remarkably, *Kras*^{G12D} activation in adult *Pdx1*-expressing cells causes an early appearance of ductal and acinar structures inside the islets of Langerhans. These ductal lesions become elongated and produce mucin resembling PanIN lesions.

4 Pancreatic Cancer Stem Cells

Irrespective of the still-ongoing debate about the cell-of-origin in PDAC, increasing evidence suggests that cells with stemness features, also termed cancer stem cells (CSCs), exclusively drive pancreatic tumorigenesis in humans. The CSC hypothesis

is the subject of great interest within the field of PDAC as well as other malignancies, since it also provides a rationale for the phenomenon of high resistance to chemotherapy leading to relapse of disease after treatment. In this context, the biological characteristics of CSCs are consistent with findings from other solid tumors. Human PDAC CSCs are characterized by several biomarkers, are able to self-renew, and to propagate the parental tumor in transplantation assays using immunodeficient mice. Biomarkers for CSCs are crucial for their identification and their tracking during treatment, representing a novel measure of treatment response. Additionally, increased understanding of the biology of CSCs could lead to the development of new treatments specifically directed against these cells as the putative root of PDAC. Currently, pancreatic CSCs are mainly identified by flow cytometry using cell surface markers that are poorly defined and non-exclusively expressed on CSCs. To date, pancreatic CSCs have been identified and characterized using the surface markers Epithelial Cell Adhesion Molecule (EPCAM or CD326), CD44, CD24, CD133, CXCR4, and c-Met. More recently, other identification methods such as the side population assay or the ALdehyde DeHydrogenase-1a1 (ALDH1) activity assay have emerged. CSC can also be functionally enriched by their capability to form spheres in vitro.

It is important to note; however, that CSCs do not necessarily represent *bona fide* stem cells nor do they necessarily arise from tissue stem cells, but rather cancer stem cells have acquired certain traits of stem cells allowing them to indefinitely self-renew and give rise to their respective differentiated progenies. While cancer stem cells share several signaling pathways that are regularly operative in normal stem cells (Micalizzi et al. 2010), they are obviously distinct from normal stem cells in terms of their in vivo tumorigenicity defined as the generation of malignant lesions upon transplantation into secondary hosts (Alison et al. 2011). Still, while it has been shown conclusively that cancer stem cells bear cell-intrinsic stemness features, they are also a product of their relationship with the tumor microenvironment affecting their aggressiveness, metastatic activity and drug resistance (Lonardo et al. 2012; Sainz et al. 2014). Thus, in order to advance our understanding of cancer stem cell biology and to develop clinically meaningful cancer stem cell-centered treatment strategies, these cells need to be studied in the context of their niche. Clinically it is of utmost importance that cancer stem cells have been proven to be highly resistant to current standard of care such as chemotherapy and radiotherapy, which makes them a probable cause of tumor recurrences after treatment (Noman et al. 2011). Consistently, primary tumors with a more prominent stem cell signature are associated with adverse outcome including higher rates of metastasis (Dalerba et al. 2011; Merlos-Suarez et al. 2011; Pece et al. 2010).

Identifying the most appropriate model systems for studying CSCs represents another important challenge for the field. The process of isolating putative CSC populations from resected tumors, whether for studying in vitro behavior or in order to obtain single cells for further analysis, is potentially prone to artifacts. Tumor digestion consists of mechanical and chemical disruption that can be harsh on the cells, impairing their viability. Therefore, the cells of interest may be lost and/or damaged during the isolation process. Moreover, modifications in cell behavior and

marker expression are to be expected due to changes in the CSC environment. Once isolated, CSCs may lose their properties due to lack of interaction with the stromal environment or with circulating stromal or endothelial cells. Furthermore, the low incidence of CSCs requires sensitive techniques for their identification and isolation. The development of comprehensive and corresponding in vitro and in vivo working models that recapitulate the whole heterogeneity of the resected tumor and mimic its complex network of relationships with the surrounding environment is thus of crucial importance for study of human CSCs. These models should correlate to the in vivo situation of the patient in order to develop and test efficient therapies targeting CSC populations. In this context, a great effort has been made on the development of primary tissue xenograft models and corresponding in vitro primary cell cultures as a platform for expansion of fresh tumor samples. These xenografts were proven highly relevant for several cancers as they accurately recapitulate the features of the patient tumor, including retaining the genetic features of the tumor, faithfully maintaining the heterogeneity of tumor cell composition, and its microenvironment including the stroma. Based on the outstanding clinical relevance of the original tumor composition, tissue xenografts have become important working models for the CSC field including pancreatic CSCs. In addition, in vivo imaging constitutes an important tool in the future working systems to study CSCs. Direct visualization of CSCs using reporter constructs provides a novel opportunity for a better understanding of tumor initiation and progression in their in vivo environment. This constitutes a crucial starting point for the evaluation of the treatment response to novel targeted therapies. Therefore, this model system provides important information on tumor biology and on the role on CSCs in the tumorigenic process, with minimal artifacts and alterations in comparison with the primary tissue.

The characteristics of the CSC population could determine the response to treatment or outcomes from cancer. To support this principle a CSC biomarker is required and has to be reproducible and measurable in patient samples. Ideal markers would be those that, while the cells remain viable, could be studied in a longitudinal fashion in order to correlate the presence of CSCs and disease outcome. The identification of CSC markers fulfilling these criteria would indeed represent a major breakthrough that could allow the development of a personalized therapeutic approach to the different types of CSCs that are resistant to chemo- and radiotherapy treatments. CSCs in PDAC have been identified by a variety of biomarkers, discussed below.

4.1 $CD44^+CD24^+EPCAM^+$ Tumor Cells

CSCs in human pancreatic tumors have been first reported in 2007. Administration of $CD44^+CD24^+ESA^+$ cells into immunodeficient mice led to tumors (Li et al. 2007), with as few as 10^2 $CD44^+CD24^+EPCAM^+$ cells initiating tumors in 50 % of transplanted mice. In contrast, up to 10^4 $CD44^-CD24^-EPCAM^-$ cells were required

to detect malignant growth. PDAC CSCs only represented 0.2–0.8 % of the whole tumor. Triple positive cells display typical cancer stem cell characteristics such as self-renewal and producing tumors of similar heterogeneity compared with the parental tumor. However, this could have been flawed by contaminating stromal cells that also include $EPCAM^-$ cells, thereby affecting the tumor formation capacity of this population. Of note, CD44 and CD24 have previously been used as cancer stem cell marker in other epithelial malignancies such as the breast or prostate cancer. Therefore, these surface markers might identify tumor-propagating cells regardless of the tumor type.

4.2 $CD133^+$ Tumor Cells

In 2007, our group demonstrated in a different study the tumorigenicity of $CD133^+$ cells isolated from fresh human PDAC. These PDAC CSCs represented 1–3 % of tumor cells (Hermann et al. 2007). As few as 5×10^2 $CD133^+$ cells induced tumor formation in immunodeficient mice, while 10^6 $CD133^-$ cells were needed for the same effect. The characteristics of the newly formed tumors were similar to the parental tumor. $CD133^+$ cells produced spheres in serum-free anchorage-independent conditions. In serial transplantations, $CD133^+$ cells exhibited self-renewal in vitro and in vivo. Interestingly, a study involving 80 PDAC patients showed that cytoplasmic CD133 expression significantly correlated with patients' outcome (Maeda et al. 2008). Finally, some $CD133^+$ cells also express $CD44^+CD24^+EPCAM^+$ (ranging from 10.3 % to 37.4 %), but no population of pure tumorigenic cells could be identified. Thus, the ideal combination of cell surface markers for the identification of a pure cancer stem cell population is still required.

4.3 Other CSC Markers

To enrich for CSC, ALDH1 has been described for isolating tumorigenic cells in the human pancreatic line L3.6pl (Kim et al. 2011). ALDH1 expression is linked to poor prognosis, and $ALDH1^+$ cells are reported to be more clonogenic with higher migratory and invasive potential (Rasheed et al. 2010). Another way to identify CSCs is a fluorescent reporter system to detect proteasome activity, in which low activity of the 26S proteasome indicates CSC features (Adikrisna et al. 2012). Moreover, the receptor for hepatocyte growth factor c-Met has also been used in PDAC to identify CSC (Li et al. 2011). Along with expression of CD133 or CD44, c-met expression strongly selected for CSC as demonstrated by enhanced in vivo tumorigenicity as compared to each of the single markers. For instance, $c\text{-}Met^+CD44^+$ cells show strong tumorigenic potential in generation of subcutaneous tumors.

As can already be conveyed from this rather large, diverse and ever growing panel of markers, the development of reliable cancer stem cells biomarker profiles

for accurately and prospectively isolating viable cells at high purity represents a daunting task. While numerous cell surface proteins have been positively evaluated in certain settings, the expression levels of many of these markers can drastically change based on environmental conditions (e.g. tumor digestion, cultivation in different conditions, xenografting), in response to treatment, and their expression is neither exclusively nor reproducibly linked to a functional cancer stem cell phenotype (Lonardo et al. 2010). Thus, alternative detection and isolation methods based on functional properties of cancer stem cells would not only avoid the use of such artifact-prone surface markers but should also provide novel insights into cancer stem cell biology. Towards this end, an intrinsic autofluorescent phenotype has been identified in cancer stem cells and was subsequently established as a novel and functionally relevant tool to isolate and characterize these cells down to single cell level (Miranda-Lorenzo et al. 2014). This distinct inherent cancer stem cell property represents a novel biological feature that is traceable in real time and provides unprecedented robustness and power for the identification and purification of cancer stem cells without the use of antibodies nor any kind of manipulation, thus drastically reducing experimental errors and artifacts. While surface marker panels are regularly tested for only certain cancer types, this novel marker has already been shown to reproducibility identify cancer stem cells across many tumor types including pancreatic, breast, lung, liver and colorectal cancer (Miranda-Lorenzo et al. 2014). Thus, it has now become possible to more accurately capture the dynamic complexity of cancer stem cells.

Functional assays such as sphere-formation capacity in vitro and particularly tumorigenicity in vivo still remain the gold standard for functionally validating CSCs. It is important to note that CSCs do not represent a homogenous clonal population of cells with equal capabilities but have undergone genetic evolution during the many years of tumor development and subsequent progression. While earlier studies in pancreatic cancer already pointed towards distinct populations of CSC with distinct features including the capability to metastasize, genetic evolution has now also been shown to occur in distant metastasis of pancreatic cancer. Genomic instability is a cause for different subclones of metastasis-initiating cells, although its relation to CSC subpopulations has not been determined yet. The issue of clonal heterogeneity of CSCs has recently been comprehensively addressed in acute lymphoblastic leukemia by studying DNA copy number alteration.

These studies demonstrate that different subclones of CSCs are present in individual patients suggesting that there is not a single CSC subset with a static phenotype, but rather that clonal evolution within CSCs is a common event. Genetic instability can cause the rise of different CSC subclones originating from a common progenitor (precursor), displing different proliferative properties and invasive features. Due to selection pressure, one or several subclones may play a dominant role in the tumorigenic and/or metastasis process. The evolving concept of CSC heterogeneity also indicates that therapeutic approaches need to be designed to target and eradicate all CSC subclones in order to be clinically efficient, as spared subclones

will lead to relapse of the disease. This multiclonality may at least in part rationalize eventual relapse of the disease even though the initiating oncogenic event has been clearly defined and targeted. This may be exemplified by the high recurrence rate in patients with chronic myeloid leukemia treated with Imatinib.

4.4 Migrating CSCs and Metastasis

Metastasizing cancer cells undergo a process called EMT involving genetic and epigenetic changes. The EMT program is required for tumor cell extravasation into the circulation, and to home and colonize remote sites of the host. A potential link between CSCs and metastasis and has been suggested previously (Brabletz et al. 2005). Moreover, stem cells are enriched in EMT genes as compared to their epithelial progeny (Mani et al. 2008), while migration to the vasculature is associated both with expression of EMT genes and other characteristics of pancreatic stem cells (Rhim et al. 2012). Interestingly, tumor cells still have the same tumorigenic potential compared with other bulk tumor cells after induction of EMT, while PanIN cells following EMT being more tumorigenic.

4.5 Heterogeneity of the CSC Pool

The CSC pool is not composed of identical CSCs but is heterogeneous due to genetic and epigenetic alterations in the CSC as well as the microenvironment. Therefore, CSCs can grow into different clones with distinct invasive properties, hypoxia resistance or susceptibility to chemotherapy. Our group has shown that while human PDAC $CD133^+$ cells show improved tumorigenicity, $CD133^+CXCR4^+$ cells display highly metastatic behavior. The specific ligand for the chemokine receptor CXCR4 is stromal derived factor-1 (SDF-1), which is secreted by the bone marrow stromal cells and is known to regulate stem cell homing to the bone marrow. Moreover, SDF-1/CXCR4 signaling promotes PDAC cell migration and invasion in vitro (Li et al. Cancer Lett 2012). Following depletion of $CD133^+CXCR4^+$ cells from the CSC pool, the metastatic behavior was abrogated while tumorigenicity was unaffected (Hermann et al. 2007). Consistent with these data, numbers of $CD133^+CXCR4^+$ cells were increased in patients with lymph node metastasis as compared to patients without metastatic disease.

Collectively, signaling pathways regulating stemness and EMT such as Hedgehog, Notch, and Wnt signaling pathways seem to be closely associated (Li et al. 2012). MicroRNAs also seem to be of importance, as the EMT-associated ZEB1 repressor has been reported to inhibit microRNAs such as miR-200c and miR-203, which in turn are involved in the inhibition of stemness (Wellner et al. 2009).

4.6 Pancreatic CSC Niche

Stem cells survive in a niche, which provides favorable conditions for it to self-renew. Similarly, a tumor is governed by its microenvironment/niche, which encompasses several components such as the cancer-associated fibroblasts, CSCs, immune cells, signaling molecules, blood vessels and the extracellular matrix. The tumor stroma is composed of pancreatic stellate cells which undergo paracrine Nodal/Activin signaling, thereby forming a paracrine niche for pancreatic CSCs. Pancreatic stellate cells secrete the embryonic morphogens Nodal/Activin, and thus support the in vitro sphere formation and invasiveness of pancreatic CSCs. Hamada et al. have shown that the presence of stellate cells improved the spheroid forming ability of cancer cells, while expression of CSC related genes such as *Nestin*, *ABCG2* and *LIN28* was induced. Hence, the cross talk between the niche and the CSCs remains pivotal.

4.7 Signaling Pathways Involved in the Maintenance of Pancreatic CSCs

Since self-renewal is a common feature of normal stem cells and CSCs, it is reasonable to believe that these cells share the same signaling pathways. The following signaling pathways such as Notch, Shh and Wnt play an important role in the pancreatic CSCs. In the normal pancreas, Notch signaling controls the balance between the self-renewal and differentiation processes. Additionally, Notch signaling is important for the pathogenesis of human cancers including PDAC. Studies showed that the overexpression of Notch-1 resulted in increased clonogenicity, migration, invasion and induction of EMT phenotype in AsPC-1, a pancreatic cancer cell line (Bao et al. 2011a). Moreover, overexpression of Notch-1 resulted in a significant increase in the pancreatosphere formation which concomitantly expressed higher levels of the CSC markers, EPCAM and CD44. Bao et al. have shown that Notch-1 signaling is crucial for the acquisition of EMT phenotype (Bao et al. 2011b). Likewise, Abel et al. have demonstrated that the Notch pathway is essential for the maintenance of the pancreatic CSC population (Abel et al. 2014). Knockdown of Hes1 using shRNA and inhibition of the Notch pathway components by gamma secretase resulted in the reduction of the self-renewal capacity of pancreatic CSCs. Altogether, these studies suggest that Notch signaling is important for the pancreatic CSC formation. Hedgehog signaling pathway is essential for cell differentiation and tissue patterning events during the embryonic development of the pancreas. Among the three hedgehog genes such as Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog homolog (Dhh), Shh shows the widest range of expression. One of these three ligands binds to the receptor Patched1, which relieves inhibition of the protein

smoothened (Smo), leading to the activation of downstream targets such as the GLI family of transcription factors and PTCH. A ninefold increase in *Shh* mRNA levels has been found in $CD44^+$ $CD24^+$ ESA^+ cells when compared to unsorted pancreatic cancer cells. Sonic hedgehog- Gli signaling is identified to be essential for the pancreatic CSCs. Sulforane (SFN), an active component in cruciferous vegetables, was found to inhibit the self-renewal capacity of pancreatic CSCs by blocking the hedgehog pathway.

In addition to the above-mentioned pathways, during embryonic development the Wnt-β-catenin signaling pathway plays an important role at different stages of pancreatic organogenesis. However, inhibition of this pathway is necessary for pancreatic specification during the early endoderm development. Canonical Wnt signaling is found to be important for the progression of pancreatic cancer. It has been reported that in colorectal cancer Wnt signaling is associated with EMT by activation of the transcription factor snail (Stemmer et al. 2008; Zhou and Hung 2005). Snail interacts with β-catenin, which is required for its activation. Since EMT is a process present in CSCs these findings suggest that b-catenin may have a role in pancreatic CSCs. However, in the future more studies are required to prove the role of β-catenin in pancreatic CSCs.

Apart from the three important signaling pathways, there are other pathways, which are involved in the maintenance of pancreatic CSCs. A recent study has reported that the inhibition of mTOR pathway by Rapamycin resulted in decreased viability of $CD133^+$ pancreatic cancer cells and reduced the sphere forming ability of pancreatic cancer cells (Matsubara et al. 2013). These results suggest that the mTOR pathway is essential for the self-renewal of pancreatic CSCs. Another study claims that the NF-kB pathway is highly activated in pancreatic CSCs, as treatment with NF-kB pathway inhibitors abrogates the stem cell-like properties (Sun et al. 2013). Altogether, several signaling pathways have been identified to play significant roles in conserving the cancer stem cell phenotype in pancreatic cancer.

5 Therapeutic Implications of Pancreatic Cancer Stem Cells

Despite great efforts, pancreatic cancer continues to be one of the deadliest cancer-related diseases in the world (Philip et al. 2009). Different treatment modalities exist for PDAC, among which surgery is the mainstay of treatment, but over 80 % of PDAC patients present with local invasion and distant metastasis upon first diagnosis. Extensive efforts have been made to improve the treatment outcome of PDAC including exploring drug combination and targeted drug chronic pancreatitis, with a 10–20 year lag between the incidences of pancreatitis and pancreatic cancer Still, progress to date has been modest (Hidalgo 2010). As a consequence, there is an urgent need to supplement current therapies and to develop novel, most likely multimodal therapeutic approaches.

5.1 Chemotherapy Against CSC Specific Features

Typically, CD133^{+} CSC are resistant to standard chemotherapy for PDAC when compared to CD133^{-} cells (Hermann et al. 2007). Following therapy with gemcitabine, the numbers of c-Met^{+} CSCs may even increase. This resistance to gemcitabine could be abrogated by the c-Met inhibitor XL184 (Li et al. 2011). PDAC CSCs can also be specifically targeted by the embryonic Activin/Nodal signaling pathway that is reactivated in adult CSCs (Lonardo et al. 2011). The Activin/Nodal signaling pathway drives self-renewal of human pancreatic CSCs via Alk4/7, the TGFβ superfamily receptors for Activin/Nodal. Knockdown of this pathway decreased sphere formation in vitro and virtually abolished in vivo tumorigenicity. Combination therapy of the Nodal/Activin inhibitor SB431542 with gemcitabine and a Hedgehog pathway inhibitor targeting stromal cells succeeded in preventing relapses in human tumor xenografts in the long run.

HDAC inhibitors also represent an attractive approach to target CSCs. Here, 5-Aza-dC and SAHA reactivate miR-34a which is an effector of p53 that is downregulated in PDAC, and thereby block self-renewal and induce apoptosis (Nalls et al. 2011). Moreover, SAHA also blocks expression of EMT inducers such as Slug, Snail and ZEB1. Metformin, an oral anti-diabetic drug for type II diabetes therapy, has been reported to show anti-tumor activity in some cancers. In PDAC, metformin decreased tumor sphere formation, which was accompanied by downregulation of pluripotency-associated genes such as Oct4 and Nanog (Bao et al. 2012). Mechanistically, metformin seems to modulate microRNAs such as let-7a, miR-26a, miR-101 and miR-200b to target tumor CSCs. Additionally, inhibitors for Notch, Hegdehog, and CXCR4 have also been examined to treat pancreatic CSCs both in vitro and in vivo models (Xia et al. 2012).

5.2 Immunotherapy Against CSCs

To date, adoptive immunotherapies have undergone a revival due to immense success in hematological malignancies. Immunotherapy may also provide new therapeutic options for PDAC if directed against bulk tumor and CSCs. Due to the fact that PDAC is highly heterogeneous and contains a variety of different mutations (up to 60 different ones), single or combinational therapies targeting signaling pathways can most likely not cope with all the genetic or epigenetic changes found in PDAC. Therefore immunotherapies targeting CSC might eradicate the whole CSC as the root of the disease.

Recently, Visus et al. isolated CSCs, including PDAC CSCs, based on ALDH activity (Visus et al. 2011). In the next step, they generated in vitro ALDH1A1-specific CD8^{+} T-cells to destroy ALDH^{bright} CSCs in human tumor xenografts models and achieved reduced tumor growth and metastasis. A drawback of this strategy may be that ALDH1A1-specific CD8^{+} T-cells might also kill normal ALDH^{bright} stem cells such as hematopoietic stem cells.

Another study showed the effect of the bispecific antibody MT110 targeting the T-cell receptor CD3 complex and EPCAM, which is frequently overexpressed on the surface of PDAC cells, including CSC (Munz et al. 2009). A drawback of targeting EPCAM might be that this marker is lost when cells undergo EMT during metastasis, so that metastasized cells may not be targeted. MT110 significantly reduced the CSC population as evidenced by reduced sphere formation capacity and in vivo tumorigenicity. Currently, MT110 is investigated in a dose-escalating phase I clinical trial enrolling patients with different epithelial cancers (lung, colon, gastric). The first results appear to be promising, demonstrating low toxicity and early signs of biological activity, opening up new opportunities for patients with PDAC.

Adoptive immunotherapies using T-cells modified to express a chimeric antigen receptor (CAR-T) against a tumor cell surface antigen have shown promise in preclinical studies using murine models of PDAC (Abate-Daga et al. 2014; Anurathapan et al. 2014; Chmielewski et al. 2012; Maliar et al. 2012). CAR-T-cell therapies against human PDAC are currently being tested in clinical trials.

5.3 Other Strategies to Target CSCs

Due to the high expression of the RON receptor tyrosine kinase in $CD44^+CD24^+ESA^+$ CSC populations, doxorubicin-liposomes coated with a RON antibody improved internalization resulting in a clear decrease in CSC viability (Padhye et al. 2011). In addition, natural compounds from dietary sources represent strategies for eliminating CSCs. For example, curcumin present in curry powders and mustard or its analogue CDF has been reported to improve the sensitivity of PDAC cells to gemcitabine. In particular, increasing PTEN and miR-200 seem to mediate CDF reduced sphere-formation and tumor growth (Bao et al. 2011a). The polyphenol Resveratrol, e.g. found in red grapes, showed anti-tumoral properties in several cancers. Apart from its reported effects in glioblastoma and breast tumors CSCs, resveratrol also blocked self-renewal of pancreatic CSCs via activation of caspase 3/7 and inactivation of Bcl-2 (Xia et al. 2012). Taken together, targeting pancreatic CSCs, the bulk tumor, and the stroma will be critical to cure the disease in the soon future.

6 Conclusions and Future Perspectives

Extensive studies over the past several years revealed the importance of a small subset of cells that could sustain the tumor. Although there are several methods employed to isolate CSCs, there are limitations with each of the currently used methods. Therefore, there is a need to identify improved methods for isolating a pure CSC population. Markers such as EPCAM, CD44, CD133 and CXCR4 have been well established in pancreatic cancer but they serve as markers for other cancer cells as well. It is of utmost importance to identify specific markers, which aid in the

maintenance of pancreatic CSCs. In the past, the identification of circulating tumor cells opened a new chapter in the field of cancer. The recent identification and characterization of an intrinsic autofluorescent phenotype in CSCs in diverse epithelial cancers including pancreatic cancer may now also provide new avenues of research. It may even be employed for the detection of tumor cells circulating in the blood stream, for which new developments are also urgently needed. It may provide us with less invasive and repetitive access to CSCs, thereby hopefully facilitating the development of CSC-centered precision medicine approaches.

CSCs share some surface markers with their normal counterparts, the pancreatic tissue stem cells, but it is still unknown whether the cell-of–origin for PDAC is a normal tissue stem cell, a progenitor cell, or a differentiated exocrine or endocrine cell with acquired stem cell characteristics. In the future, specific CSC promoters will be used to drive activation of *Kras* oncogene to clarify which pancreatic cell subset derives the carcinogenic process. In contrast, the existence of pancreatic CSCs forming spheres in vitro and enhanced in vivo tumorigenicity of tumors identical to the parent cells has been confirmed.

The next milestone to deliver is the real-time observation of pancreatic CSCs in the native in vivo setting, as demonstrated for other solid tumors using clonal analysis after lineage tracing in mice (Chen et al. 2012; Driessens et al. 2012; Schepers et al. 2012). In addition, in vivo imaging of CSCs using abdominal windows might be helpful to get in-depth insights into the role of CSCs in their natural microenvironment. As evidence is now accumulating for novel therapeutic targets that are capable of eliminating CSC, newly emerging treatment regimens that include this knowledge arising from the CSC concept may eventually lead to a better outcome for patients suffering from this currently deadly disease.

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Chapter 11
Glioblastoma Cancer Stem Cells

Luca Persano, Alessandro Della Puppa, Elena Porcù, Francesca Maule, and Giampietro Viola

Abstract Many types of cancer, including Glioblastoma (GBM), contain functionally subsets of cells with stem-like properties named cancer stem cells (CSCs). These are characterized by chemotherapy resistance and considered one of the key determinants driving tumor relapse. Many studies demonstrated that glioma stem cells (GSCs) reside in particular tumor niches that are necessary to support their behaviour. Indeed, the microenvironment is essential for GBM tumorigenesis and progression, particularly for the continuous signal communications between GSCs and cells belonging to the GBM niches, like endothelium or pericytes, which give rise to a complex plasticity of the tumor. This signal integration originates numerous mechanisms which lead to resistance to therapy. Understanding the mechanism of action of the microenvironmental signals and the interplay between different cell types within the tumor mass, open new questions on how GSCs modulate GBM aggressiveness and response to therapy. The definition of these tumor features will allow to setup innovative multimodal therapies able to target GBM cells at multiple levels. In this chapter, we will discuss the major advances in the study of GSCs role in GBM and the therapeutic implications resulting from them, thus reporting the development of new targeted-therapies applied to counteract and overcome GBM intrinsic resistance to therapy which could improve the overall therapeutic ratio of conventional treatments.

Keywords Glioblastoma multiforme • Cancer stem cells • Glioblastoma cancer stem cells • Hypoxia • Vascular niche

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1 Introduction

The term 'glioma' is referred to all tumors that are thought to be of glial cell origin. As described by the World Health Organization (WHO) classification (Louis et al. 2007), malignant diffuse gliomas are comprised of astrocytic, oligodengroglial, and mixed oligoastrocytic neoplasms based solely on morphology and are further subdivided by tumor grade based on additional histological features in the tumor. Nuclear atypias and mitotic activity are required criteria for grade III lesions, and the presence of necrosis or microvascular proliferation is required for the diagnosis of grade IV astrocytomas, named glioblastoma. Glioblastoma (GBM) is the most common and lethal primary malignant brain tumor. Together with grade III anaplastic astrocytoma, these tumors embrace the clinical entity termed "malignant glioma."

Extensive genomic characterization has recently provided a high resolution picture of the molecular alterations underlying this tumor providing the emerging view that "GBM" represents several histologically similar but molecularly heterogeneous diseases, thus influencing classification systems, prognosis, and therapeutic decisions. GBM represents the most common primary intrinsic malignant brain tumor diagnosed each year in the United States; there are ~10,000 new diagnoses annually, and >50,000 patients are currently living with the disease (Dolecek et al. 2012). All gliomas are more common in men than in women. GBM is associated with the highest median age at diagnosis. Examination of brain tumor incidence data from CBTRUS for the 10-year period from 1985 to 1994 revealed a slight but statistically significant average annual percentage increase in incidence (0.9 %). It is likely, however, that most, if not all, of this increase is attributable to improvements in diagnostic imaging and increased availability of medical care and neurosurgeons. While 90–95 % of GBM arise de novo and are considered "primary," about 5–10 % arise from lower-grade gliomas in younger patients and are termed "secondary" (Ohgaki and Kleihues 2005). Although many risk factors for developing GBM have remained unidentified, risk factors such as exposure to ionizing radiation have proven to be detrimental for disease development in some cases. Other risk factors including cell phone use, head trauma, and pesticide exposure have yet to be confirmed as increasing risk for gliomagenesis. Symptoms of disease depend on the specific location of the tumor, and diagnosis is most commonly made following surgical resection. The prognosis for patients with GBM is often very poor (only 2 % of patients aged 65 years or older, and only 30 % of those under the age of 45 years at diagnosis, survive for 2 years or more), and treatments to cure this cancer have yet to be devised.

The clinical hallmarks of GBM are its aggressive growth and inexorable recurrence despite multimodal therapy with surgery followed by radiation and temozolomide (TMZ) therapy. Unfortunately, current standard-of-care therapy results in a median survival of only 12–15 months (Stupp et al. 2005). Consequently, our present strategy is to identify genetic, behavioral, environmental and developmental contributors to glioma risk through epidemiological studies, with the ultimate goal of reducing the disease burden.

2 Emerging Role of Glioblastoma Stem Cells and the Therapeutic Challenges

GBM is a highly heterogeneous tumor with individual histologic hallmarks including high cell density, intratumoral necrosis, vascular hyperplasia and invasion through brain parenchyma (Westphal and Lamszus 2011). This heterogeneity is also displayed at the microscopic level, where the cellular hierarchy has been demonstrated to be governed by the presence of GSCs (Dirks 2008; Ignatova et al. 2002). The clinical implications of CSC targeting to improve treatment of GBM could be remarkable. Since GBM presents different phenotypic patterns and molecular signaling activation in distinct regions (layers) of the tumor mass, the pathological characterization can be influenced by the site of sample collected by the surgeon throughout the tumor (Pistollato et al. 2010). Indeed, O(6)-methylguanine-DNA methyltransferase (MGMT) has been found differentially expressed among the three layers, and both MGMT protein expression and promoter methylation status are considered important prognostic factors (Della Puppa et al. 2012; Stupp et al. 2005). This issue is crucial because in the modern neuro-oncological setting, several diagnostic and prognostic markers are commonly analyzed to predict tumor grade and the consequential therapeutic approach. In addition, biomarkers are pivotal in the selection of glioma patients for their recruitment into clinical trials following surgery. In this sense, site of the tumor sample collection could represent a remarkable bias for both selection and stratification of patients.

Current treatment of GBM is based on surgery, followed by radio and chemotherapy. In GBM surgery, intra-operative targeting of CSCs should be a main purpose. Indeed, being putative CSCs considered the major responsible of resistance requiring supplementary treatments, surgeon should achieve the complete removal of CSC population (Rampazzo et al. 2014). Currently, no techniques aiming at this purpose are available.

A further consideration can be done about loco-regional therapies, which are treatments that surgeons can carry out directly in the surgical cave after tumor removal. This is the case of carmustine (bis-chloroethylnitrosourea, BCNU or BiCNU), an alkylating agent, wafers that are a worldwide approved treatment for both newly diagnosed and recurrent high-grade gliomas. They are constituted by degradable biopolymer wafers impregnated of BCNU that is released over few weeks in the surgical cave. Wafers are implanted in the surgical cave after tumor removal, and positioned in tight contact with the brain surface infiltrated by tumor. When a complete removal of central core of tumor has been achieved, loco-regional therapy such as BCNU wafers could be more effective against a limited CSC population. However, the residual GSCs might be targeted by using pro-differentiating treatments together with conventional therapies, thus affecting CSC phenotype and aggressiveness (Persano et al. 2012). During GBM management, surgery is followed by radiotherapy and concomitant alkylating agents based chemotherapy that could be virtually more effective against a tumoral residue possibly depleted of CSCs (Pistollato et al. 2010).

3 Glioblastoma Stem Cells

In the adult brain, neural stem cells (NSCs) were observed at any stage of the development, from the embryo to the adult organism. NSCs are located primarily in the subventricular zone (Altman 1965), in the subgranular zone and the dentate gyrus of the hippocampus (Altman and Das 1965). In particular NSC have been described to reside in their specific niches around the blood vessels where they are in communication with other cells and the extracellular matrix. Different cellular types are present in these niches, such as neuroblasts, and transitory amplifying progenitors and all these cells are surrounded by ependymal cells (Facchino et al. 2011; McLendon and Rich 2011). NSCs are pluripotent cells capable of differentiation as a result of which they lose their stem properties (Schiffer et al. 2010). Moreover, their proliferative capacity and the association with blood vessels stimulate NSCs to migrate and invade surrounded tissues. While NSCs are necessary for a correct neurological development and activity, cells with aberrant NSC characteristics have been often correlated to brain tumors. Indeed, increasing evidences suggest the existence of a population of CSCs or tumor initiating cells (TICs) with high self-renewal ability, promoting brain tumor growth, in contrast to the other cancer cells (Persano et al. 2011).

In the light of the "CSC hypothesis", the transformation of NSCs or progenitors in CSCs follows the rules of the normal physiology but with aberrant order, timing and intensity of the underlying mechanisms. CSCs may originate from normal NSCs undergoing tumorigenic alterations. Differently, they can derive from more differentiated or terminally differentiated transit-amplifying neural cells being affected by multiple mutations, thus reverting to a stem phenotype. Moreover, an arrest of the normal maturation process of the NSC has been also reported, thus leading to intensive cell division and lack of differentiation. CSCs originating through these different processes are generally described as a small sub-population of dividing cells with stem cell-like properties, huge self-renewal ability, peculiar genetic alterations, tumorigenic potential, and the ability to differentiate into all different bulk tumor cells (Vescovi et al. 2006).

The first evidence of the existence of cells with stem-like characteristics in GBM was reported by Steindler and colleagues, who isolated clonogenic, neurosphere-forming precursors from post-surgery specimens of human GBM (Ignatova et al. 2002). At a later stage, two independent groups demonstrated that GBM and medulloblastoma contain neurosphere-forming cells that are able to give rise to neuronal and astroglial-like cells (Lee et al. 2006; Singh et al. 2003, 2004). GBM cells need specific criteria to be classified as GSCs. In particular, they should be able to self-renew, differentiate into distinct lineages and initiate tumors in immunodeficient animal models, recapitulating the original phenotype and heterogeneity of the parental tumor (Singh et al. 2003, 2004). The presence of these cells in GBM specimens was observed by culturing GBM tissues in serum-free media supplemented with EGF and bFGF growth factors, which formed non-adherent spheroids with an enhanced GSCs population. Neurosphere cultures are currently the most common

method used to propagate GSCs in vitro. It has been demonstrated that these neurosphere cultures maintain genetic profiles similar to the original GBM patients and form invasive tumors in intracranial xenografts (Ernst et al. 2009; Lee et al. 2006; Singh et al. 2004). Each neurosphere arises from an individual GSC or transit-amplifying cell and despite their clonal origin, neurospheres are heterogeneous aggregates that consist of GSCs, transit-amplifying cells and more differentiated GBM cells. When these neurosphere cultures are dissociated to single cells, a small proportion of them can give rise to secondary neurospheres (Chen et al. 2010; Reynolds and Weiss 1996). In contrast when they are exposed to fetal bovine serum, neurosphere originating cells differentiate into the different cell lineages of the parent tumor (Singh et al. 2003). Thus, GSCs show high capacity to proliferate, self-renewal properties and the ability to form secondary neurospheres. Moreover, GSCs significantly differ from NSCs for their ability to differentiate and then revert to the original stem/immature phenotype. Indeed, differentiation induced by serum of normal NSCs is permanent (Lee et al. 2006), while glioma lines established by serum cultures are reversible and they can be converted to neurospheres when cultured in serum-free media (Gilbert et al. 2010; Qiang et al. 2009).

GBM tumor mass consists of different cell phenotypes, requiring the individuation of specific markers to more precisely identify GSCs. GSCs are expected to share common markers with their normal counterparts showing usually elevated expression of Nestin, an intermediate filament expressed in NSCs, located in neurogenic niches (Reynolds and Weiss 1992; Uhrbom et al. 2002) and correlated with 'stemness' and cytoskeleton organization, cellular signaling, organogenesis and metabolism. During the differentiation process NSCs lose the expression of Nestin and start to express βIII-tubulin and glial fibrillary acidic protein (GFAP) (Jackson and Alvarez-Buylla 2008; Sequerra et al. 2013). GSCs have been reported to show increased GFAP expression, a marker of astrocyte differentiation that can be co-expressed similarly to Nestin by NSCs. GSCs are also enriched for Sox2, a transcription factor expressed by NSCs with cytoplasmic localization which is connected to the differentiation process and associated with multipotency and pluripotency (Ikushima et al. 2009, 2011). Comparative gene expression analysis led to identification of more GSC markers, including Oct4, SSEA-1/ CD15, Bmi-1, Musashi-1, Nanog, integrin-α6, L1CAM, A2B5 and ABC-type transporters (Gonzalez-Gomez et al. 2011; Ikushima et al. 2011; Son et al. 2009). However, the marker which is commonly used to identify and isolate GSCs is CD133 (also known as Prominin-1), a 5-TM glycoprotein expressed by human hematopoietic cells and neural progenitor cells (Pfenninger et al. 2007; Wang et al. 2008). In the human fetal brain, CD133 is a marker for NSCs (Uchida et al. 2000) and its expression has also been observed in intermediate radial glial cells in the early postnatal brain, and in ependymal cells in the adult brain (Coskun et al. 2008; Pfenninger et al. 2007). $CD133^{+}$ cells from GBM are capable of multi-lineage differentiation and have a high capacity to form neurospheres, unlike the corresponding $CD133^{-}$ cells which did not proliferate in neurosphere cultures. In addition, $CD133^{+}$ cells from GBM have an increased capacity of tumor initiation after serial transplantations in immunodeficient mice (Singh et al. 2004).

The GSCs biology is influenced by various signaling pathways that maintain self-renewal or regulate differentiation in the appropriate context. The group of Fine started culturing tumor cells in serum-free conditions (Lee et al. 2006). By using EGF and FGF, we can reduce cell differentiation and promote GSC self-renewal. These mitogens act through their receptor tyrosine kinases (RTKs) inducing activation of downstream pathways such as the phosphoinositide 3-kinase/Akt (PI3K/Akt) and Mitogen-Activated Protein Kinase (MAPK), leading to cell proliferation, survival and tumorigenicity (Hambardzumyan et al. 2008a, b; Lee et al. 2006).

Originally identified as a regulator of neurogenesis, Notch signaling plays a central role in nervous system development, including maintenance of self-renewal ability and regulation of fate decisions into neural and glial lineages (Artavanis-Tsakonas and Simpson 1991; Yoon and Gaiano 2005). Upon binding to its ligands (Delta-like and Jagged), heterodimeric Notch receptors (Notch1–4) get cleaved by γ-secretase in the cytoplasm, releasing the Notch intracellular domain (NICD). NICD translocates into the nucleus where it acts as co-activator for the transcriptional repressors of neurogenic genes, such as Hes and Hey, sustaining stemness in activated cells (Mizutani et al. 2007). In GBM, Notch signaling is involved in several distinct mechanism in tumorigenesis, through the regulation of both self-renewal and differentiation of GSCs (Hovinga et al. 2010; Lino et al. 2010; Wang et al. 2010). Furthermore, Numb, which prevents NICD from traveling to the nucleus and thus inhibits downstream signaling upon Notch activation, was shown to be asymmetrically distributed within GSCs and to promote asymmetric division, giving rise to a stem cell and a more restricted and differentiated cell (Jiang et al. 2012).

Transforming growth factor-β (TGF-β) signaling promotes GSC self-renewal through regulation of distinct mechanisms. In particular, it was shown to act through SRY-Related HMG-Box transcription factors Sox2 and Sox4, to induce self-renewal (Ikushima et al. 2009).

Sonic Hedgehog (Shh)-Gli signaling is highly important for brain and spinal cord patterning during embryonic development and plays crucial functions in GSC maintenance (Cayuso et al. 2006; Shahi et al. 2008). It has been shown to promote GSC self-renewal and expression of stem cell genes, whereas its blockage leads to apoptosis, delay in tumorigenesis and inhibition of GSC self-renewal and migration (Bar et al. 2007; Rossi et al. 2011; Ulasov et al. 2013).

The Wnt/β-catenin pathway induces proliferation and/or differentiation of progenitor cells within gliomas and it is important for GSC self-renewal. Moreover, overexpression of Wnt ligands, Wnt3a and Wnt1, has been observed in GSCs (Kim et al. 2012; Rampazzo et al. 2013).

Bone morphogenetic protein (BMP), a member of TGF-β superfamily, functions as a differentiation signal within GBM, as opposed to the previously discussed roles of other members of the TGF-β family in maintenance of self-renewal (Ikushima et al. 2009). The difference between BMP and TGF-β effects on GSC biology can be owed to distinct signaling cascades, even though they belong to the same superfamily of ligands. Recent evidences suggested that Notch signaling is also important for transdifferentiation of GSCs into tumor-derived endothelial cells (Wang

et al. 2010). Similarly, TGF-β was shown to induce GSCs differentiation into vascular pericytes, supporting vessel formation and leading to further tumor growth (Cheng et al. 2013; Wang et al. 2010).

4 Glioblastoma Microenvironment

GBM complexity is driven by numerous stimuli which originate from the microenvironment, important for pathogenesis and resistance to therapy. It has been described that GBMs display high cellular heterogeneity, and Pistollato et al. (2010) described a model which integrates the plethora of signals which regulate GBM plasticity.

GBM cells communicate with the perivascular niche and with the hypoxic niche, by originating a "teamwork", withstanding to hierarchic rules and complex networks. The three-layers concentric model represents a clear explanation to elucidate the complexity of signals integration in GBMs, particularly deriving from microenvironment (Fig. 11.1). According to the hierarchical theory for tumor progression, the "tumor-initiating cells" GSCs should originate from the sub-ventricular zone (SVZ) and the sub-granular zone (SGZ), which include progenitor cells able to originate multilineage differentiated cells. These specific niches are essential for maintaining stemness and self-renewal properties of GBM precursors, which are secondly instructed to proliferate and differentiate.

The central area of the tumor mass consists of a necrotic core, highly hypoxic and enriched in GSCs, and as going to the periphery, the tumor mass includes an intermediate layer, hypoxic and rich in GSCs too. The surrounding peri-tumor zone corresponds to the peripheral layer of the "three-layer model", and it is highly vascularized and presents few GSCs and more differentiated cells (Fig. 11.1). A hypoxic gradient is arranged from the core to the periphery, associated to a progressive change in the expression of specific markers, from stemness markers, like CD133 and Nestin in the necrotic area, to differentiation markers, such as GFAP and β-III-tubulin, in the more oxygenated periphery.

Two main niches are detected in GBM microenvironment, the hypoxic and the perivascular ones. They finely regulate cellular fate by releasing numerous stimuli, which promote cell differentiation or stemness maintenance. GBMs are highly vascularized tumors, characterized by strong angiogenesis, but the blood flow is not the only determinant factor to have a pivotal role to contribute to the complexity of vascular microenvironment, since many cell types infiltrate the tumor mass. Precisely, the perivascular niche consists of the surrounded area of angiogenic and tumor microvascular structures, characterized by the presence of several mature and differentiated cells (endothelial cells, fibroblasts, astrocytes, macrophages or microglia) which orchestrate intercellular crosstalk. Endothelial cells are the principal component of the vascular niche, and they differ from endothelial cells which constitute vessel walls. Blood flow is necessary to provide oxygen and nutrients to GBM cells, particularly to CSCs, nevertheless many non-structural endothelial cells

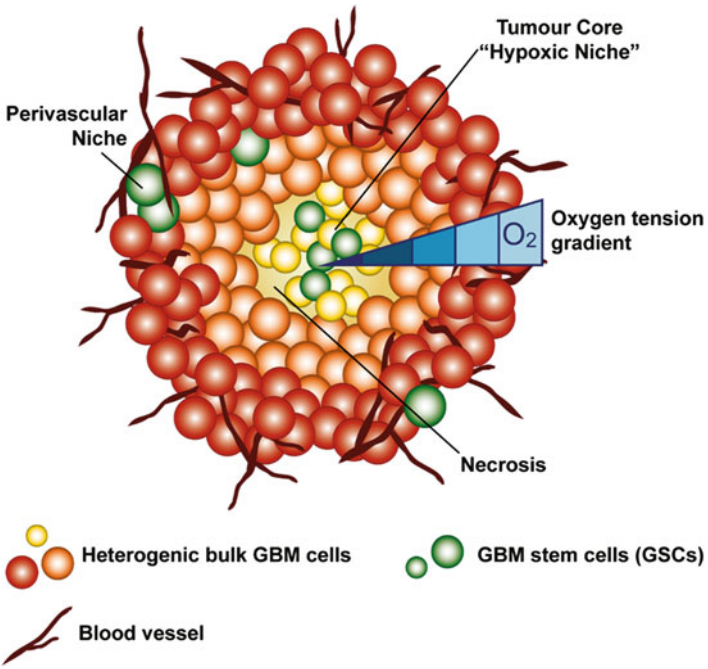

Fig. 11.1 *The three layer model of glioblastoma.* In this model GSCs are located along the hypoxic gradient in the tumor mass, mostly residing in the inner portions of the mass and in the so called perivascular niche. The GBM cells derived from the inner areas of the mass are resistant to chemotherapy in vitro. Accounting for the heterogenic landscape of genetic and genomic aberration characterizing GBM cells isolated GSC from the tumor core and the perivascular niche of the GBM mass are characterized by a different phenotype and tumorigenic potential. Cytogenetic analysis demonstrated that the two types of GSCs bear quite different genetic abnormalities, nevertheless deriving at least in part from common precursor cells. A hypoxic gradient is present from the tumor core to the periphery, associated to a progressive change in the expression of specific markers such as stemness markers, like CD133 and Nestin in the necrotic area, to differentiation markers, such as GFAP and β-III-tubulin, in the more oxygenated periphery

exist, and they remain separate from tumor capillaries, without increasing the tumor microvascular density. They have the task of releasing a lot of diffusible factors to maintain the self-renewal ability of neural stem cells and neurogenesis. On the other hand, GBM cells release pro-angiogenic stimuli like VEGF to recruit endothelial cells which proliferate and give rise to new capillaries. Moreover other pro-angiogenic mechanisms were described for GBM angiogenesis, such as the transdifferentiation of cancer stem cells into tumor-derived endothelial cells (TDECs), to continuously preserve the vascular microenvironment (Calabrese et al. 2007; Soda et al. 2011; Charles and Holland 2010).

Pericytes are contractile cells which are tightly associated to endothelial cells, to stabilize and maintain the integrity of the newly formed tumor vessels. They has been described to be involved in the regulation of the angio-architecture structural shape of the tumor vascular niche, and they intimately depend on endothelial cells

along the vessel walls. Analogously, astrocytes are closely associated to the endothelial cells forming blood vessels, and they both maintain the integrity of the blood brain barrier, and produce neurotrophic factors which promote GBM proliferation (Hoelzinger et al. 2007).

Fibroblasts reside in the perivascular niche, and they are responsible of GBM invasion, as reported for other cancer types. They express critical markers associated to tumor progression and malignancy, such as metalloproteases (pro-MMP2).

The presence of tumor induces a physiological immune response, and GSCs showed the expression of pro-inflammatory genes, which stimulate the enrichment of microglia at the tumor perivascular site. Microglia are the macrophages which lie in brain tissue, and they are the principal cytokine stimulators important for tumor proliferation, migration and progression. They are located in many sites, depending on their role. They promote metastasis when arranged in the perivascular space, cell motility and invasion when sited in the advanced tip of tumor, and their localization in the perinecrotic area increases angiogenesis, explaining the positive correlation between macrophages infiltration and vascular density in gliomas (Nishie et al. 1999; Roggendorf et al. 1996).

The combination of all these cell types results in a complex system of crosstalk between cells, which culminates in a fine balance of a plethora stimuli for GBM cells. Particularly GSCs are strictly connected to endothelial cells, as well as other stromal cells, defining the entirely plasticity, typical of the tumor microenvironment. It has been observed that GSCs arrange themselves along the capillaries, in order to be prone to respond to signaling cues deriving from endothelium, by direct cell-to-cell contact and soluble factors. They stimulate GSCs to proliferate and self-renew, and the increase of the number of endothelial cells has been associated to an accelerated brain tumor initiation and growth. On the other hand, GSCs express elevated levels of VEGF or other pro-angiogenic factors, which in turn stimulate endothelial cells to proliferate and undergo angiogenesis. This evidence shows a bidirectional signaling and cross-talk between stem cells and vascular niche (Charles and Holland 2010).

A peculiar aspect of GBM microenvironment is the hypoxic niche. GBM mass is characterized by low oxygen concentrations, ranging between 0.1 % and 2.5 %, unlike in healthy brain which physiologically range between 12.5 % and 2.5 % of oxygen. GBMs are marked out by hypoxic gradients, which present areas with moderate or severe hypoxia, and necrotic zones in the tumor core. The inner layer shows a considerable expression of hypoxic markers, associated to tumor aggressiveness and GSCs maintenance. The milestone of hypoxia are HIFs, a family of transcription factors which response to oxygen tension and regulate hypoxia responsive genes, playing a pivotal role in cancer progression, metastasis and resistance to therapy. HIFs consist of two subunits, HIF-α and HIF-β, which form a functional heterodimer acting as nuclear transcription factor in hypoxic conditions. Normoxia induces HIF-α hydroxylation providing its proteasomal degradation. Human HIF-α consists of three oxygen-sensitive subunits, HIF-1α, HIF-2α, HIF-3α. HIF-1α is the most ubiquitously expressed, and the mostly studied. HIF-2α is predominant in GSCs niche, unlike HIF-3α which does not work as transcription factor as lacking

the transcriptional activation domain, but it acts as dominant negative by sequestrating HIF-β. This subunit is not responsive to oxygen concentration, and it is constitutively expressed in all cell types (Yang et al. 2012). HIF-1α and HIF-2α are important to determine a switch to an acute response to hypoxia, mediated by HIF-1α, and a chronic reaction principally regulated by HIF-2α (Koh and Powis 2012). HIFs are involved in several processes since they regulate both normal tissue homeostasis and disease progression. HIF controls metabolism, induces angiogenesis and stemness maintenance, it is involved in tumor initiation and progression and stimulates tumor invasion (Majmundar et al. 2010).

The putative CSCs are preserved in the hypoxic niche, since HIF promotes an undifferentiate state in populations of progenitors and stem cells. It has been shown both in vitro and in vivo that HIF depletion in $CD133^+$ GSCs impairs their ability to induce angiogenesis and tumorigenesis (Li et al. 2009).

HIFs can transcribe for more than 40 target genes (Semenza 2002), among which the carbonic anhydrase isoform 9 (CAIX), involved in increasing the metastatic potential of GBM by acidification of the tumor microenvironment, and Notch1, which leads to NFAT activation and cell proliferation and tumor growth. Thus hypoxia sustains GBM cells proliferation, particularly preserving the stem population in the perivascular and hypoxic niches, by up-regulating other transcription factors like Notch and Oct4, which control self-renewal and multipotency of stem cells. Moreover, it has been described that HIF counteracts the differentiating stimuli induced by BMPs (Pistollato et al. 2009). In vitro hypoxia stimulates both the expression of the stem markers CD133, Nestin, Sox2, and the formation of neurospheres, characterized by elevated stem potential (Bar et al. 2010; Harris 2002; McCord et al. 2009). HIF is directly engaged in angiogenesis and tumor invasion, by activating several factors such as VEGF, metalloproteases, TGF factors and CXCR4 (Kaur et al. 2005).

GBM microenvironment is essential for GBM tumorigenesis and progression, particularly for the continuous signal communications between GSCs and cells belonging to the GBM niches, like endothelium or perycites, which give rise to a complex plasticity of the tumor. This signal integration originates numerous mechanisms which lead to resistance to therapy. HIF expression is associated to drug resistance and poor patient prognosis in multiple tumor types, and in GBM hypoxia has been shown to mediate both radiotherapy and chemotherapy resistance. Indeed, in hypoxic conditions the radiation dose required to have the same biological effect as in normoxic conditions is three times higher (Spence et al. 2008). Moreover, traditional chemotherapy for GBM and specifically treatment with TMZ is impaired by signals induced by the hypoxic niche, resulting ineffective (Persano et al. 2012; Pistollato et al. 2010). This phenomena are explained by the observations that low oxygenation induces significant changes in the expression pattern of genes and proteins which are related to the regulation of DNA-damage response, apoptosis and proliferation.

Since the strict connection of GSCs and GBMs niches, several therapeutic targets have been found among the signaling molecules deriving from the microenvironment stimuli. Clearly HIF-1α has been identified as the principal target for

improving GBM therapy, with the strategy to reduce its mRNA/protein levels, to impair the interaction with HIF-β or with DNA at the transcription sites, or to increase the protein degradation. Secondly, GBM vascular compartment and endothelial cells are other important targets with the purpose to reduce the nutrients supply of tumor. Nevertheless, in order to design combination treatment, recent evidences demonstrated the importance of the correct timing of treatment, to facilitate the delivery of chemotherapeutics into the tumor mass, and successively deplete the tumor vasculature. The principal molecular targets of the vascular niche are VEGFs and PDGFs signaling pathways. Moreover, other signal cues may be arrested, such as chemokines associated to tumor migration and invasion of surrounded tissues, among which the more relevant is CXCR4.

In conclusion, the complex integration of signals deriving from GBM niches is necessary for GBM tumorigenesis and aggressiveness, and to regulate the whole network of stimuli deriving from several cellular types present in the microenvironment. They regulate stem cells fate or their maintenance, having a pivotal role in GBM progression and resistance to therapy. Only considering GBM cells together with stem cells in strict contact to the microenvironment will lead to optimize winning therapeutic strategies for GBM.

5 Therapeutic Targeting of Glioma Stem Cells

Neuro-oncology has experienced an explosion in the molecular modeling of GBM through tumor genetics and mouse modeling. The Cancer Genome Atlas (TCGA) confirmed the frequent mutational involvement of the p53, RB, and receptor tyrosine kinase (RTK) pathways in GBM (Cancer-Genome-Atlas-Research-Network 2008). Moreover, gene-expression studies divided GBM patients into distinct tumor subtypes – classical, mesenchymal, neural and proneural, each characterized by a peculiar mutational load in epidermal growth factor receptor (EGFR), neurofibromin 1 (NF1), platelet-derived growth factor receptor A (PDGFRA) and Isocitrate Dehydrogenase 1 (IDH1) (Verhaak et al. 2010). Although this large scale effort suggested a number of possible GBM targets, only few of these genetic findings have entered into clinical practice to date (Yan et al. 2013).

As previously outlined, GBM display high resistance to conventional radiotherapy and chemotherapy (Sanai and Berger 2008). Indeed, soon after their initial description, GSC resistance to treatments have been described (Bao et al. 2006; Liu et al. 2006), thus suggesting them as one the principal contributors to GBM tumor recurrence. GSCs have been demonstrated to be more resistant to radiation than the non-stem glioma cells (Bao et al. 2006). Indeed, chemotherapy with TMZ delays GBM tumor growth, but long term survivors are extremely rare and recurrence after TMZ therapy strongly indicates the presence of TMZ-resistant GSCs (Stupp et al. 2005). In an in vivo mouse model of GBM, TMZ treatment increased tumor side population (SP), a cell population that have been described to be enriched in CSCs, suggesting that TMZ treatment could even favor tumor recurrence (Chua et al.

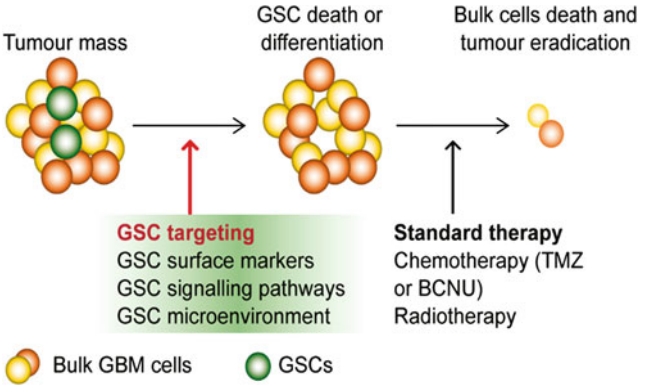

Fig. 11.2 *Targeted therapy in Glioblastoma.* Conventional therapies (surgery, radiotherapy, chemotherapy) target the tumor bulk, but display no efficacy toward the GSC compartment. Microenvironmental factors and activation of specific signaling pathways are able to sustain the little population of remaining GSC, allowing for GBM relapse. Latest studies have been trying to generate new targeted therapies (*green box*) able to differentiate or eliminate the GSCs or signals from the microenvironment able to maintain this cell pool

2008). For these reasons, it is now widely accepted that GSCs contribute to GBM recurrence after conventional therapies. Thus, there is a urgent need to develop more effective therapies based on the specific targeting of signaling pathways involved in the maintenance of GSCs functions (Fig. 11.2).

Initial models of GSC regulation have been based on neural stem cell (NSC) biology, the probable normal cellular correlate. Despite, GSCs seem to be governed by pathways active in brain development, including Notch, Wnt, bone morphogenetic protein (BMP), transforming growth factor-β (TGF-β), and RTK pathways (Binda et al. 2014), our knowledge of the mechanisms underlying GSC maintenance and resistance to therapy are still in early development, thus preventing their complete understanding. Moreover, recent evidence support the idea that using GSC enriched cell cultures derived from human GBM biopsies could be a better strategy to setup more appropriate drug discovery programs, although with some caveats in terms of inter – and intra-tumoral GSC heterogeneity, their isolation and proper long term expansion (Romaguera-Ros et al. 2012). Despite these limitations, potentially important therapeutic targets in GSCs have been published on a frequent basis. Here, some of previously identified GSC targets and possible novel therapeutic strategies against them are discussed (Fig. 11.2).

5.1 Targeting GSC Surface Molecules

Based on the suggestive but still debated hypothesis that a unique surface marker expression would be able to define the entire GSC population (Perez Castillo et al. 2008), one target of particular interest to the field is CD133. A functional role for

CD133 has been reported in GSCs and other tumors, as regulator of the PI3K–Akt pathway via its interactions with the p85 subunit of PI3K (Wei et al. 2013) and consequently involving Erk1/2 and MAPK signaling (Dong et al. 2010). Upstream of this cascade, RET has been identified as a crucial mediator of CD133 intracellular functions in neuroblastoma cells (Takenobu et al. 2011). As a cell surface protein, CD133 has been targeted with antibodies in preclinical studies and a vaccine against CD133 (ICT-121) is entering clinical trials (Yan et al. 2013). Moreover, direct targeting of GSCs cell surface molecules has been investigated by a lentiviral preparation expressing a shRNA for L1 cell adhesion molecule (L1CAM), a molecule preferentially expressed in $CD133^+$ GBM cells, which is able to suppress GBM cell growth in vitro and in vivo (Bao et al. 2008).

5.2 Overcoming Radiation and Drug Resistance

DNA repair mechanisms can restore the integrity of damaged DNA bases and thus contribute to drug and radiation resistance. In this context, cancer stem cells have been reported to possess enhanced DNA repair capacity (Johannessen et al. 2008). One of the first studies in this field was published by Rich's group, reporting that CD133 cells survived ionizing radiation in greater proportions compared to cells that lacked CD133 expression (Bao et al. 2006). This effect has been associated to the over-activation of Chk1 and Chk2 DNA damage checkpoint kinases in the $CD133^+$ GSC population. In fact, conventional radiation is able to exert phosphorylation of these cell cycle effectors in $CD133^+$ cells, but not in $CD133^-$, suggesting a constitutive activation of multiple cell cycle checkpoints in GSCs that may further up-regulate in response to DNA damage (Nakai et al. 2009). Chk1 and 2 activation can be inhibited by a specific inhibitor debromohymenialdisine (DBH) representing an intriguing target for GSC treatment (Bao et al. 2006).

Resistance of $CD133^+$ GSCs is also probably sustained by the combined higher expression of drug resistance, DNA repair enzymes and anti-apoptosis proteins such as breakpoint cluster region pseudogene 1 (BCRP1), *O*-6-methylguanine-DNA methyltransferase (MGMT) and FAS-associating death domain (FADD)-like antiapoptotic molecule (FLIP), respectively (Liu et al. 2006). In this context, our group previously reported that O(6)-benzylguanine (6-BG), a nontoxic pseudosubstrate inhibitor of MGMT, treatment is able to sensitize GSCs to chemotherapy with TMZ (Pistollato et al. 2010).

This high resistance of GSCs to radiation and anticancer drugs has been investigated by many authors and associated to both DNA repair and non-DNA-repair mechanisms including heat shock protein-90 (HSP-90) inhibition, synergizing with radiation and/or TMZ (Sauvageot et al. 2009), treatment with anti epidermal growth factor receptor (EGFR) antibodies (cetuximab and nimotuzumab), able to increase radiosensitivity (Michelakis et al. 2010) or blockade of chloride transport, enhancing chemotherapy-mediated cell death (Kang and Kang 2008).

5.3 Targeting GSC Signaling Pathways

Self-renewal and survival of Neural Stem Cells (NSCs) are mainly regulated starting from embrional development by both the Notch family proteins and by epidermal growth factor (EGF)-activated signaling pathways (Aguirre et al. 2010). In particular Notch pathway activation is the primary responsible for NSC maintenance and differentiation inhibition, whereas EGFR sustains proliferation and migration of newly derived precursors from NSCs. Thus, maintenance of the balance between stemness and differentiation can result from the dynamic interplay between Notch and EGFR pathways.

5.4 Notch

Similar to what happens during normal neural development, it has been documented that Notch is a critical regulator of CSC maintenance in several types of tumors, including GBM. Fan et al. showed that Notch blockade by γ-secretase inhibitors reduced neurosphere growth and clonogenicity of GSCs in vitro (Chen et al. 2010; Fan et al. 2010; Ulasov et al. 2013). Moreover, Notch blockade has been correlated to GSC chemotherapy sensitization and to inhibition of xenograft recurrence (Gilbert et al. 2010). Hovinga et al. also emphasized that the Notch pathway plays a critical role in linking angiogenesis and CSC self-renewal and thus is a potential therapeutic target (Hovinga et al. 2010). Also Notch ligands such as Delta-like Ligand 4 (DLL4) have been associated with tumorigenesis and GSC maintenance (Li et al. 2011). Overall, the inhibition of Notch signaling should be considered as a promising therapeutic target for GSCs.

5.5 EGFR and PI3K/AKT

EGFR is overexpressed and/or mutated in many carcinomas, including lung, breast, colon, head and neck, prostate, ovarian, but displays some specific mutations also in GBM (Inda et al. 2010). PI3K/Akt/mTOR pathway, being aberrantly activated by EGFR amplification or the presence of the EGFRvIII ligand-independent variant, is thus often up-regulated in GSCs (Bleau et al. 2009), conferring them survival and/or proliferative advantages. The targeting of this important signaling cascade at different levels (by blocking EGFR, PI3K or directly AKT) might overcome the unsatisfactory results observed in clinical studies when RTK inhibitors have been used alone (Florio and Barbieri 2012). Particularly interesting are results obtained with A-443654, able to inhibit GSC proliferation in vitro and in vivo (Gallia et al. 2009) and the combination between the mTOR inhibitor temsirolimus and perifosine (Pitter et al. 2011). Recent findings from Kitanaka's group suggest that PI3K/Akt/

mTOR and MEK/ERK pathways coordinately regulate the differentiation and tumorigenicity of GCSs. Also in this case, concomitant inhibition of both pathways more potently suppress their survival signals rather single inhibitions (Sunayama et al. 2010). In this study FoxO3a was reported as fundamental for the differentiation of GSCs induced by Akt and Erk inhibition and that its constitutive activation is sufficient to induce differentiation and to inhibit GSC self-renewal and tumorigenicity, suggesting that FoxO3a may be a potential therapeutic target (Persano et al. 2013; Sunayama et al. 2011). Finally, knockdown of CD133 in GSCs causes down-regulation of Akt phosphorylation, highlighting the strict link between stem cell surface markers and activation of intracellular signaling (Eyler et al. 2008; Gallia et al. 2009).

5.6 Shh

Sonic hedgehog (Shh)-Gli signaling is another of the key regulator pathway in the NSC niche during embryogenesis (Binda et al. 2014) and, being critical for NSC maintenance is often aberrantly activated in GBM thus supporting GSC growth and maintenance (Clement et al. 2007). Indeed the potent Shh antagonist cyclopamine depletes GSCs, reducing self-renewal and the tumorigenic potential of GBM stem cells, increasing also TMZ and radiation-mediated cell death (Bar et al. 2007; Merchant and Matsui 2012). Clinical trials with another Shh signaling antagonist, vismodegib, are ongoing in comparison with standard chemotherapy (Lorusso et al. 2011).

5.7 Ephrins

Of the numerous receptors that have been implicated in GSC biology, the Ephrin (Eph) RTKs have been investigated in cancer and stem cell biology. Indeed, they regulate a wide range of physiological processes in the CNS during development and, in adult neurogenesis, they affect NSCs survival and proliferation (Depaepe et al. 2005; Holmberg et al. 2005; Pasquale 2008). Recently, it has been shown that GSCs are a major site of EphA2 overexpression and that EphA2 expression correlates with both the size and tumorigenic potential of the GSC pool. Furthermore, forced down-regulation of EphA2 expression suppresses GSC self-renewal and intracranial tumor-initiating ability, showing that this receptor may represent a selective molecular target for potential therapeutic purposes (Binda et al. 2012, 2014). Moreover, EphA3 was found to be specifically expressed in mesenchymal GSCs and appeared to modulate downstream mitogen-activated protein kinase (MAPK) signaling, thus appearing as another possible Eph signaling target (Day et al. 2013).

5.8 Induction of Differentiation

Differentiation therapy forcing GSCs to differentiate might be a promising and notably non-cytotoxic strategy for GSC targeting. In this regard BMPs may be potential soluble factors in the treatment of gliomas (Persano et al. 2012). BMPs are members of the Transforming Growth Factor-β (TGF-β) family of ligands, but exerting opposite effects. In fact, TGF-β has been shown to have regulatory effects on GSC differentiation by inducing the Smad-2⁄3 transcriptional complex thus preventing GSC, but not normal neural stem⁄progenitor cells differentiation (Penuelas et al. 2009).

The prototypic receptors for BMP in mammals are the type II receptor, BMPR2, and type I receptors, BMPR1A and BMPR1B (Chen and Panchision 2007). It has been reported that BMP2 and 4 act as neuroepithelial proliferation/differentiation signals at different stages of embryonic central nervous system development, an effect mainly mediated by BMPR1A and BMPR1B respectively (Chen and Panchision 2007). For this reason, BMPs have been used as pro-differentiating factors for GBM treatment. Despite, we and others recently reported on the role of BMPs, in particular BMP2 and BMP4, in promoting astroglial differentiation and in reducing cell growth of GBM-derived cells (Persano et al. 2012; Piccirillo et al. 2006), considering BMPs treatment a promising therapeutic approach for brain cancer, enthusiasm has been weaken by a study showing that GSC may epigenetically reduce BMPR1B expression thus evading BMP-induced differentiation (Binello and Germano 2011; Lee et al. 2008).

Recently, Chirasani et al. clearly demonstrated in vivo and in vitro that BMP7, another member of the bone morphogenetic protein family, released by neural precursor cells induces differentiation and represses proliferation, self-renewal and tumor initiation of GSCs (Chirasani et al. 2010). Moreover, a BMP7 variant have been shown to inhibit GBM growth in vitro and in vivo (Tate et al. 2012).

These results suggest to explore further if the inhibitory effects mediated by BMPs on cell growth are targeted specifically on the CSC population, and whether other soluble factors are useful to selectively inhibit cancer stem cells growth. Overall, mimicking events induced by BMP2,4,7 and their effectors remains a potential important therapeutic tool and clinical trials using BMPs are being designed.

For further information, we report also treatment with all-trans retinoic acid (ATRA), Interferon-β (IFN-β) and agonists of peroxisome proliferator-activated receptor (PPAR) c as all able to induce GSC differentiation with different mechanisms involving activation of nuclear retinoic acid receptor (RAR) and STAT-3 signaling pathway respectively (Campos et al. 2010; Chearwae and Bright 2008; Yuki et al. 2009).

6 Conclusion and Future Perspectives

A great number of advances have been made in trying to setup better therapeutic strategies for GBM patients care. The rise of models explaining GBM origin and progression by the involvement of GSCs, and their sharing by the scientific community has led, in the recent years, to a downright explosion of interest in this field, also rising some concerns about the real efficacy of standard treatments applied for GBM. TMZ chemotherapy, despite introducing a real increase of patients' survival, is nevertheless based on an old concept of anti-cancer drugs targeting highly proliferating cells. Indeed, TMZ is a DNA alkylating agent able to effectively get through the blood–brain barrier, that, since it is orally administered, highly meets with patients compliance. The high rate of relapse after surgery, radiation and chemotherapy raises the consciousness that these standard treatments are still not sufficient. Thus a novel class of drugs is urgently needed to overcome GBM intrinsic resistance to therapy. Although many compounds demonstrated strong efficacy in preclinical studies, none or only few of them showed similar effects during clinical trials, due to negligible anti-tumoral activity or severe side effects. This could be due to the GBM tumor intrinsic heterogeneity and for this reason a better understanding of GSCs behavior, phenotype and signaling activation status must be improved. Thus, future therapies should be validated on GSCs rather than cell lines. Next years will be fundamental to validate recent developed agents or novel delivery strategies for future patients care, trying to counteract this almost incurable disease.

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Chapter 12
Head and Neck Cancer Stem Cells

Chiara Bianchini and Andrea Ciorba

Abstract Head and neck cancer is still the sixth most common cancer type worldwide. Surgery, radiotherapy and chemotherapy, alone or in combination, are still considered the main therapeutic approaches. Disappointingly, despite significant advances in head and neck treatments, survival rates and prognosis have only moderately improved through the years. Understanding the biological mechanisms of cancer initiation, development and spreading, represents a primary purpose in order to eradicate the disease; it is essential to find out the cellular/molecular factors that can be involved in the head and neck cancerogenetic process. In particular, the existence of cancer stem cells has been matter of discussion and a number of articles have been published about the role that these cells play in tumor biology, particularly among development and maintenance of cancers. Aims of this chapter are to discuss about: (i) the recent advances in understanding the molecular mechanisms at the basis of cancer initiation and progression in Head and Neck, particularly examining the possible role of stem cells in cancer occurrence and progression in Head and Neck cancers; (ii) the identification of cellular/molecular indicators of malignant conversion and progression; (iii) the possible therapeutic implications/development of targeted cellular strategies. Molecular/cellular targeted therapies could offer increasingly customized solutions based on the identification of multiple specific pathways essential for cancer development and metastasis. Based on these observations, the molecular recognition of cancer progenitor cells and/or cancer stem cells must be considered for improving the efficacy of the current cancer therapies. Additional investigations could help us in better understanding the origin and the molecular behavior of head and neck cancer, therefore leading us to program and perform a more specific, safe, effective, "personalized", "targeted" therapeutic plan.

Keywords Cancer stem cells • Head and neck cancer • Cancer stem cell markers • Head and neck cancer biomarkers

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1 Head and Neck Cancer Biology and Pathology

Head and neck cancer is reported to be the sixth most common cancer type worldwide (Braakhuis et al. 2005; Forastiere et al. 2001; Bianchini et al. 2008). The incidence of Head and Neck squamous cell carcinoma has been reported to be of 780,000 new cases per year over all the world. Major risk factors for Head and Neck tumors are tobacco and alcohol abuse (Mannelli and Gallo 2012). Interestingly, human viruses, such as papillomavirus (HPV) and Epstein-Barr virus (EBV), have been claimed to be critically involved in the carcinogenesis of some Head and Neck tumors. In particular, regarding HPV infection, HPV 16 and 18 seem to be the most prevalent subtypes found within neoplastic cells in almost 50 % of oropharynx cancers. However, the molecular mechanisms of this virus-related carcinogenesis require further studies (Syrjänen 2005; Stransky et al. 2011).

Men are usually more affected than women even if in the last years the incidence ratio is varying because of the increase of smoking habit in women (Mannelli and Gallo 2012). Concerning age, patients are usually older than 40 years old in 98 % of cases and the median age at diagnosis is 60 in western world (Mannelli and Gallo 2012; Sturgis et al. 2004; Dobrossy 2005). Several clinical and pathological prognostic factors have been proposed for Head and Neck tumors. Nonetheless, most of them retain a low sensitivity and accuracy and thus cannot be considered as a safe and reliable guide for the treatment decisional process (Ginos et al. 2004; Mannelli and Gallo 2012). Disappointingly, during the last years, despite the advances in surgical and other treatments, which have also aimed to improve quality of life, survival rates have not grown significantly. The presence of metastases as well as the developing of therapy-resistance, local and regional recurrences maintain mortality rate of this disease still very high (Mannelli and Gallo 2012). Moreover, the significant morbidity subsequent to treatment often require long term multidisciplinary care (Braakhuis et al. 2005; Forastiere et al. 2001; Bianchini et al. 2008).

2 Cancer Stem Cells in Head and Neck Tumors

Highly tumorigenic, stem-like cells have been demonstrated in Head and Neck squamous cell carcinomas firstly by the Prince's laboratory in 2007 (Prince et al. 2007). Tumor cells were isolated for the expression of the $CD44^+$ cell surface marker and tumor initiation was therefore noted in immunodeficient mice inoculated with these cells. Following this milestone work, other laboratories confirmed that in Head and Neck squamous cell carcinomas, $CD44^+$ cells fit the definition of cancer stem cells (Sterz et al. 2010).

Recent studies of cell lines derived from oral squamous cell carcinoma indicate the presence of several subsets of cells with phenotypic and behavioural features corresponding to cells capable of commencing tumors *in vivo* (Lapidot et al. 1994; Bonnet and Dick 1997; Costea et al. 2006; Chiou et al. 2008). Thus, in human

laryngeal tumors, Chinese investigators have characterized a cluster of $CD133^+$ stem cells of the Hip-2 cell line, suggesting that this subset population could retain a strong selected tumorigenic ability (Wei et al. 2009). A population of highly malignant cells in a head and neck squamous cell carcinoma cell line has also been individuated from primary head and neck cancers using xenotransplantation techniques. These cells named SASVO3, possess enhanced tumorigenic ability both *in vitro* and *in vivo*, and exhibit properties of stemness, including abilities of sphere-forming, potential of transplanted tumor growth and elevated expression of stem cell markers such as $CD133^+$ and/or Bmi1 (Chen et al. 2009a).

Also, Suer et al. (2014) have isolated stem-like cells from freshly resected laryngeal tumor specimens and have characterized them by quantitative real time PCR; cancer stem cell markers including $CD133^+$, SOX2, OCT4, KLF4, ABCG2, CXCR4 (which have been reported to be associated with resistance of tumors) have been isolated. Similarly, Han et al. (2014) have identified a small set of $CD24^+/CD44^+$ cells in head and neck squamous cell carcinoma that show high stemness characteristics of self-renewal and differentiation.

Tumor development is related to local growth and to the spreading of cluster of cells within adjacent and distant tissues. Particularly concerning tumor progression, it has been proposed that, once a cancer stem cell acquires one (or more) genetic alteration, then forms a patch in the mucosal epithelium with genetically altered daughter cells. As result of this process, cancer stem cell escapes the normal control mechanisms and gains growth advantage. As the patch starts to expand, the tumor develops and starts spreading. In this way, areas of normal epithelium can be replaced by cell populations that become progressively more genetically aberrant. The presence of a large number of genetically altered cells is considered as a serious risk and at a certain time this process can lead to the development of a malignant clone (Bianchini et al. 2008; Forastiere et al. 2001).

As another model of cancer stem cell progression, 'perivascular niches' have been claimed to highly contribute to cancer spreading and there is evidence among the existence of a supportive perivascular niche in head and neck cancer. These clusters of cells have been reported to be a complex environment where intricate interactions among cells and matrix components allow stem cell survival, within solid tumors such as glioblastoma (Ritchie and Nör 2013; Borovski et al. 2011). In particular, it has been described that the majority of the cancer stem cells are found within a 100 μm radius of blood vessels and that this niches could also be found in human head and neck squamous cells carcinoma. The identification of these niches could be of particular interest for the comprehension of tumor biology and for the detection of new possible targets (Ritchie and Nör 2013; Borovski et al. 2011).

Indeed, the identification of cancer stem cells in head and neck tumors represents a fundamental goal in the agenda of stem cell biologists as well as the detection of the key factors involved in self-renewal and differentiation pathways. The biomolecular markers defining cancer stem cell subpopulations could be an optimal target, even if they show a large variance that makes difficult their characterization and the study of the tumor biology (Mannelli and Gallo 2012).

3 Putative Cancer Stem Cell Markers in Head and Neck Cancer Stem Cells

Cancer stem cells could be responsible not just of tumor initiation but also of aggressive tumor behaviours such as metastasis, chemoresistance and radioresistance (Mannelli and Gallo 2012). Understanding the molecular biology of cancer stem cells would help, not only in identifying the mechanism of tumorogenesis, but could also offer the possibility to detect cellular indicators of malignant conversion and progression (biomarkers), that could help us in early identification of cancer stem cells in a specific tissue. This could represent a powerful tool for early diagnosis of particular interest for clinical practice (Ailles and Prince 2009; Mannelli and Gallo 2012). So far, few reliable markers have been identified in head and neck tumors (Table 12.1).

3.1 CD133⁺

CD133 is a cell surface antigen, also known as Promin 1 or PROM 1. This is a transmembrane glycoprotein, already characterized as a possible marker of cancer stem cells. In some Head and Neck squamous cell carcinomas, $CD133^{+}$ cells were found to have increased clonogenic activity when compared to $CD133^{-}$ cells (Ritchie and Nör 2013). Oral cancer stem-like cells from cell lines and primary tumors have been found to have an increased expression of CD133 and these cells have been related to have an increased activity to form clones and invasiveness (Wei et al. 2009).

3.2 CD44⁺

CD44 is another cell surface glycoprotein that is kreported to be involved in cellular cross-talk, cell-adhesion and migration. CD44 has been implicated in head and neck tumor progression and chemoresistance and local aggressiveness. This has also

Table 12.1 Putative cancer stem cells markers in head and neck cancers

Markers	Tumor type	Reference(s)
CD 133+	Head and neck squamous cell carcinoma	Wei et al. (2009)
CD 44+	Head and neck squamous cell carcinoma	Ailles and Prince (2009)
ALDH1	Head and neck squamous cell carcinoma	Chen et al. (2009b), Clay et al. (2010)
MPP-9	Head and neck squamous cell carcinoma	Sterz et al. (2010)
GDF 15	Nasopharyngeal carcinoma	Chang et al. (2007)
Oct4	Head and neck squamous cell carcinoma	Koo et al. (2014)
BMI1	Head and neck squamous cell carcinoma	Allegra et al. (2014)
CD166	Head and neck squamous cell carcinoma	Yan et al. (2013)
c-Met	Head and neck squamous cell carcinoma	Sun and Wang (2011)

been the first marker described within head and neck cancer stem cells (Ritchie and Nör 2013; Prince et al. 2007; Gao et al. 2011; Wang and Bourguignon 2011).

3.3 Aldehyde Dehydrogenase

Aldehyde dehydrogenase (ALDH) has been considered as a biomarker for cancer stem cells. Even if its role in head and neck squamous cell carcinoma has yet to be determined, it has been reported that $ALDH1^+$ cells show radioresistance and represented a reservoir for tumor growth. Recent evidence supports the use of $ALDH^+$ as a single marker to identify cancer stem cells in HNSCC (Ritchie and Nör 2013; Clay et al. 2010; Chen et al. 2009b; Chang et al. 2007).

3.4 Other Putative Biomarkers

Sterz et al. (2010), using immunohistochemical analysis, found antigens CD44 and MMP-9 to co-localize tumor cells at the invasive front within head and neck squamous cell carcinomas; particularly at the western blot analysis they pointed to a role of a MMP-9 positive basal-cell-like cell layer in the process of HNSCC invasiveness. Oct4 has also been reported to be a critical regulator of stemness in head and neck squamous carcinoma cells, as cancer stem cells from squamous cell carcinoma expressing high levels of Oct4 have been shown to retain more stem cell-like traits, such as self-renewal, stem cell markers expression, chemoresistance, invasion capacity and xenograft tumorigeneity *in vitro* and *in vivo* (Koo et al. 2014). Emerging studies also show that the oncoprotein BMI1 (B-cell-specific Moloney murine leukemia virus integration site 1) to be an important function as a biomarker of cancer stem cells, also in Head and Neck cancers (Allegra et al. 2014). See Fig. 12.1.

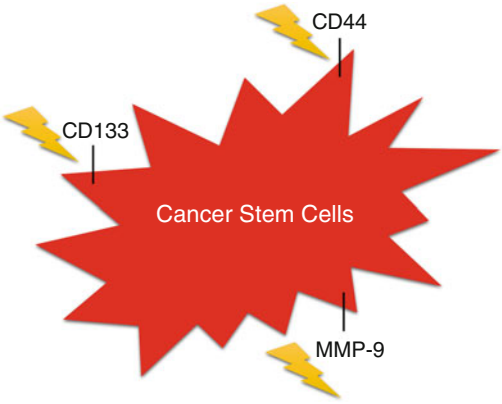

Fig. 12.1 Possible sites of intervention for identifying (and hopefully arresting) head and neck cancer stem cells progression

4 Clinical and Therapeutic Implications

The ability in identifying cancer stem cells should lead towards a more specific tumor treatment and eventually to tumor prevention. Different etiological factors and risk habits can result in distinct genetic and epigenetic alterations, which may activate different signaling pathways, thus impacting differently on the development and progression of Head and Neck tumors (Bianchini et al. 2011; Matta and Ralhan 2009; Lamont et al. 2001; Ganly et al. 2000; Ziober et al. 2010). It is clear that, the discovery of specific initial biomarkers could offer promising results; the early detection of Head and Neck tumors represent a key point in increasing treatment success (Mannelli and Gallo 2012; Bianchini et al. 2011).

Surgery, radiation, chemotherapy, and combinations of these approaches, are currently used in the management of head and neck cancer patients. Since the presence of cancer stem cells with the bulk of a tumor could explain the occurrence of treatment failure, the ability in identifying cancer stem cells could allow a more specific and 'tailored' oncological treatment. Future goal will be to possibly identify therapeutic molecular targets of cancer stem cells in order to attack specifically cancer stem cells only, within the bulk of the tumor or even metastatic cells (Tirino et al. 2009; Mannelli and Gallo 2012; Bianchini et al. 2011).

The first successful targeted therapy (EGFR-specific antibodies) demonstrates that improved understanding of the molecular pathways underlying Head and Neck squamous cell carcinoma could help in setting valuable new treatment protocols. So far, the most promising and advanced therapeutic strategies, clinically available, aim to (i) block growth factor based cellular signaling and (ii) interfere/block cancer angiogenesis related pathways. Particularly, the molecular targeting of developmental cascades, defined of therapeutic interest, include hedgehog pathway, Wnt/catenin, Notch, EGFR, PDGFR and KIT pathways and/or oncogenic signaling elements (telomerase, Src, ABL, PI3K/Akt, MYC, NF-κB and survivin), which assume a critical function in regulating the self-renewal, survival and invasion of cancer progenitor cells as well as in drug resistance and disease relapse (Mannelli and Gallo 2012; Bianchini et al. 2011; Mimeault and Batra 2006, 2007a, b; Dean et al. 2005; Barker and Clevers 2006; Rubin ad deSauvage 2006; Roberg et al. 2007; Fodde and Brablets 2007; Zhang et al. 2007; Mimeault et al. 2008).

Probably, a combination of different protocols that target different but specific pathways is likely to inhibit the escape of cancer stem cells towards an uncontrolled growth and spreading, therefore leading to a more effective disease control (Mannelli and Gallo 2012; Bianchini et al. 2008; Bianchini et al. 2011). Possibly, in the future, the identification of biomarkers could allow us to (i) avoid cancer spreading by identifying micrometastasis or even the ability of a cancer to metastatize; (ii) identify resistance to chemo- or radio-therapy and cancer susceptibility to different therapies. Tailored treatments represent indeed a future goal for cancer therapy also in Head and Neck tumors.

5 Conclusions and Future Perspectives

The development of new effective and safe targeted therapies for eradicating all cancer progenitor cells, as well as their further differentiated progenies, at the primary tumor site and at metastatic sites, hopefully should allow us, in the future, to enhance the available cancer treatments, prevent the disease recurrence and therefore obtaining a complete clinical and 'cytological' remission and cure of cancer patients (Bianchini et al. 2011).

Even if we are still a long way from understanding the molecular mechanisms that guide carcinogenesis, and therefore from applying them to clinical protocols, the develop of new *in vitro* and *in vivo* models of cancer stem cells could offer new insights in order to develop more successful strategies also for head and neck cancer treatment. At the same time, the cancer stem-cell model could hopefully have an impact also on earlier cancer detection, by the identification of new molecular markers of malignancy. In our opinion, (1) the further clarification of the critical molecular events involved in head and neck carcinogenesis, as well as (2) the identification of new cellular/molecular markers, are possible accessible targets among head and neck cancer research in a near future.

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Chapter 13
Leukemic and Lymphoid Stem Cells

Michael W. Becker and Kristen M. O'Dwyer

Abstract Cancer cell heterogeneity is a feature of nearly all cancers and can be related to three major influences: (1) genetic and epigenetic heterogeneity due to clonal evolution/collapse; (2) microenvironmental influences; and (3) the underlying tissue hierarchy from which the tumor arises. Ongoing studies in whole genome sequencing and of the bone marrow, splenic and lymph node microenvironments demonstrate their contributions to tumor heterogeneity. In this chapter we will focus on the role of the hematopoietic hierarchy in blood cancer cellular heterogeneity; one of the most studied systems in mouse and human. Observations that myeloid and lymphoid malignancies harbor rare relatively quiescent therapy resistant cell populations date back over 30 years. Early studies in chronic myelogenous leukemia were consistent with a disease origin in the hematopoietic stem cell and subsequent studies have confirmed these findings. The publication by Bonnet et al. in 1994 offered the first prospective assessment of human cancer stem cell populations and established acute myeloid leukemia (AML) as a model system. In the intervening years, new technologies have allowed a continued reassessment of cancer stem cell populations in AML, myelodysplastic syndromes, multiple myeloma and acute lymphoblastic leukemia. While these studies have confirmed the existence of the rare cells capable of recapitulating the malignancy on transplantation, they have also identified considerable inter- and intra-patient heterogeneity with conflicting results on the ability to identify potential cancer stem cells using surface antigen profiles alone. Novel xenotransplantation models, whole genome sequencing and other technologies offer the tools to further refine this model in hematologic malignancies and develop rational therapies to target leukemia stem cells.

Keywords Hematopoietic stem cells • Leukemic stem cells • Pre-leukemic stem cells • Chronic myelogenous leukemia • Acute myelogenous leukemia • Acute lymphoblastic leukemia • Multiple myeloma • Myelodysplastic syndromes

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1 Introduction

Normal hematopoiesis relies on a highly regulated multi-tier hierarchy with a pool of rare, largely quiescent hematopoietic stem cells (HSCs) at the base (Rieger and Schroeder 2012; Morrison and Weissman 1994). Downstream are oligo-potent, lineage restricted progenitors with a capacity for robust proliferation in response to stress and terminally differentiated functional elements (Fig. 13.1). In hematopoiesis, the capacity for self-renewal is confined to mainly quiescent populations and is tightly regulated through cell autonomous and non-autonomous signals. Malignancies of the hematopoietic system are similarly heterogeneous and studies over several decades have consistently demonstrated that the capacity to repopulate the tumor on serial transplantation is confined to a rare population of cancer cells with the capacity to self-renew. Chronic phase CML is characterized by a limited mutation burden, a consistent leukemic stem cell (LSC) phenotype and an effective

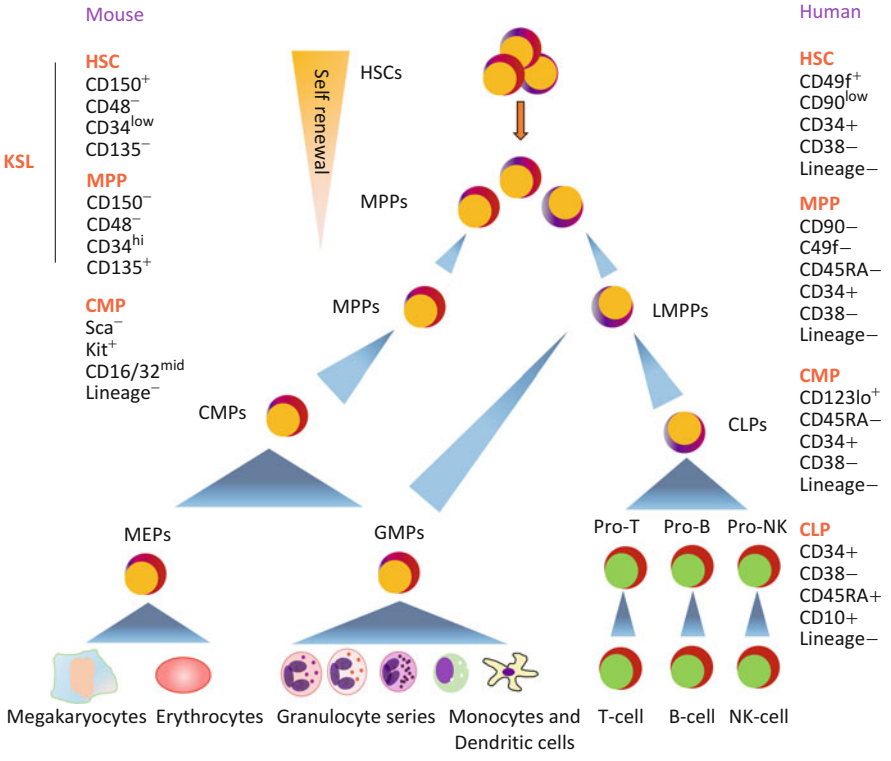

Fig. 13.1 Summary of the stages of normal hematopoiesis and their surface antigen phenotype(s) for HSCs and early progenitors for both mouse and human

therapy. In contrast, acute myelogenous leukemia (AML), myelodysplastic syndromes (MDS), B-cell acute lymphoblastic leukemia (B-ALL) and multiple myeloma (MM) result from multiple genetic and epigenetic events with increasing diversity in the cancer stem cell phenotypes. Studies of healthy aging donors as well as patients in remission have identified some of the potential early events and have identified possible limitations of targeting CSCs. Adding to the complexities, studies in human B-cell malignancies call into question the unidirectional nature of transitions between levels of the hierarchies.

2 Hematopoietic Stem Cells and Normal Hematopoiesis

2.1 Assays for the Study of Normal and Malignant Hematopoiesis

Study of normal and malignant hematopoiesis has been aided by a number of assays (Table 13.1). Commercially available standardized reagents and protocols permit comparison of results across labs. Historically suspension culture of HSC and early progenitor pools for more than a few days led to irreversible commitment and loss of stem cell function. Novel in vitro culture conditions extend the period of time prior to loss of HSC function and allow HSC expansion (Delaney et al. 2010). An adaption of the methylcellulose colony forming assays involves serial replating of methylcellulose colonies and serves as a surrogate marker for proliferative capacity with the number of replatings before CFU activity is extinguished related to self-renewal capacity. Cobblestone area forming (CAFC) and long term culture initiating cell (LTC-IC) assays allow the study of ST-HSC, MPPs and CMP/CLPs in vitro by co-culture with defined stroma. The readout for these assays is either scoring the number of CAFC beneath the stromal cell layer or transfer of hematopoietic cells into defined media methylcellulose to quantitate CFU activity. The CAFC assay has been adapted to allow large scale in vitro screening of potential LSC targeting molecules (Hartwell et al. 2013).

Table 13.1 List of assays commonly employed in the study of normal and malignant hematopoiesis

Assay	Cell population applicable
Immune phenotyping	All levels
Serum free suspension culture	HSC, progenitor level
Methyl cellulose/CFU assay	Progenitor level: multipotent or lineage restricted
LTC-IC/cobblestone forming assay	ST-HSC, MPP, CMP, CLP
Syngeneic/xenotransplantation	Lt-HSC, ST-HSC, MPP
Limiting dilution analysis (LDA)	HSC or LSC frequency

2.2 *Identification of Normal Murine and Human Hematopoietic Stem Cells*

A major strength of studying normal hematopoietic stem cell populations in mice is the availability of congenic strains that allow tracking donor and recipient contribution to the peripheral blood, spleen and marrow following transplantation. The C57bl/6 strain remains the standard for studying hematopoiesis. The cumulative results of mouse transplantation studies have resulted in a defined surface antigen phenotype that permits the transplantation of a single cell with hematopoietic reconstitution (Kiel et al. 2005) (Fig. 13.1).

Early models for human AML xenotransplatation suffered from an inability to work with small cell numbers, the need for cytokine supplementation and the impact of residual immune function. Increasingly immune deficient strains of mice have revolutionized the study of both normal and malignant hematopoiesis (Ito et al. 2002; Shultz et al. 2005). The NOG/NSG and also the NOD/SCID ß2m^{null} mice are among the most immune deficient strains of inbred laboratory mice described to date and demonstrate greater permissiveness in human tissue engraftment. These strains have been compared head to head with the NOD/SCID strain and permit engraftment of a greater percentage of primary AML patient samples as well as allow a higher level of engraftment. Direct transplantation of cells into the femur of the recipient further increases the efficiency of transfer (McKenzie et al. 2005). It is now possible to perform single cell xenotransplantation studies of normal human HSCs (Notta et al. 2011). Models attempting to maximize the capacity for human xenotransplantation studies in NOD, C57BL/6 and Balb/c strains are ongoing (Iwamoto et al. 2014). As xenotransplantation models improve results from prior LSC studies are being re-evaluated with regard to the impact of the assay on the observed phenotypes.

Application of the aforementioned assays has allowed careful delineation of the hematopoietic hierarchy in mouse and man. While an in depth review of normal hematopoiesis is outside the limits of this chapter several concepts are worth mentioning. First, the HSC pool size and makeup are tightly regulated through cell autonomous as well as extrinsic mechanisms many of which are still poorly understood (Fig. 13.1). There remains a mostly unexplained age dependent heterogeneity in the HSC pool with variation in the composition, kinetics, and progeny outputs when analyzed using clonal marking studies (Jordan and Lemischka 1990; Dick et al. 1985) or transplantation of highly purified populations (Uchida et al. 2003; Ema et al. 2005). Second, the assays employed to characterize stem cell and progenitor populations remain dependent upon the demonstration of a capacity for tissue regeneration following transplantation. The increasingly recognized heterogeneity of the populations in Fig. 13.1 and the instability of the available phenotypes have impaired efforts to circumvent the need for functional validation, i.e. transplantation.

3 The Role of Malignant Stem Cells in Myeloid Malignancies

3.1 Leukemic Stem Cells in CML

Chronic myeloid leukemia (CML) is a myeloproliferative disorder that can exist in three distinct phases, chronic phase, accelerated phase, and blast crisis, which is phenotypically identical to acute leukemia. The defining lesion of this disease, the Philadelphia chromosome (Ph), encodes the BCR/ABL proto-oncogene and results in a constitutively active protein tyrosine kinase product, p210BCR-ABL (Nowell and Hungerford 1960; Rowley 1973; de Klein et al. 1982). This event targets the normal HSC compartment resulting in inappropriate expansion of the granulocytic lineage. The LSC has been studied extensively in all phases of this disease as well as in the remission state i.e. minimal residual disease (MRD). Hence, CML is a near perfect paradigm for understanding the leukemia stem cell model.

In 1977, Philip Fialkow and colleagues provided some of the first data that CML is a clonal stem cell disease (Fialkow et al. 1977). They studied glucose-6-phosphate dehydrogenase (G-6-PD) isoenzymes in the granulocytes of eight woman with CML, and utilized X-linked polymorphisms to compare the enzyme types found in skin cells, normal granulocytes, and CML granulocytes. They found that the patients were heterozygous at the X-linked G-6-PD locus in the skin cells, but homozygous for G-6-PD enzyme type in the CML cells, as well as in the erythrocytes, platelets, and macrophages. This data suggested that these cells were derived from a common stem cell, i.e. the normal HSC compartment. Since this initial discovery and the works of others, the CML stem cell phenotype in chronic phase has now been defined as $CD34^{+}CD38^{-}CD90^{+}Lin^{-}Thy1^{+}$ (Ahuja et al. 1989; Jorgensen and Holyoake 2007; Eisterer et al. 2005; Holyoake et al. 1999).

Jamieson and colleagues evaluated samples from blast crisis CML to demonstrate that in CML blast crisis, secondary genetic events target the cell populations with surface antigen and gene expression profiles resembling a committed progenitor, $CD34^{+}$, $CD38^{+}$ $lineage^{-}$, and result in the acquisition of unlimited proliferative and self-renewal capacity, mostly through activation of the ß-catenin-signaling pathway (Jamieson et al. 2004). In a subsequent report, Abrahamsson et al. (2009) further identified glycogen synthase kinase 3beta missplicing events as contributors to expansion of self-renewal capacity to the GMP population. Follow-up studies have also identified a role for other mutations and pathways in the transition from chronic phase CML to blast crisis.

The CML blast crisis model suggests that while the “first hit” (i.e. acquisition of the oncogene BCR-ABL), targets the normal HSC, secondary genetic events target the committed progenitor pool and result in unlimited proliferative and self-renewal potential. The secondary events that occur in blast crisis CML are the first clear evidence from human cancer that secondary events can instill the critical stem cell property of self-renewal in a population of cells that is normally non-self-renewing.

The CML stem cell has been studied in the minimal residual disease state as well. Targeted therapy with tyrosine kinase inhibitors (TKIs), such as Imatinib, Dasatinib, Nilotinib, induces complete cytogenetic responses {i.e. no evidence of the (Ph) chromosome by FISH analysis} in more than 80 % of patients (Druker et al. 1996, 2006). The BCR-ABL transcript can still be detected by RT-PCR in most patients, however. In patients with undetectable transcript levels, approximately 60 % of patients will relapse when TKI therapy is discontinued (Rousselot et al. 2007). These clinical observations suggest that a quiescent LSC exists in CML and is resistant to TKI therapy and thus represents a reservoir for relapse of the disease. Corbin and colleagues examined how the LSC and progenitor cells survive during Imatinib therapy and asked whether the LSC is BCR-ABL dependent or independent. Utilizing a series of phosphorylation assays; the group demonstrated that the CML stem cells are not dependent on BCR-ABL activity for survival, and persist in patients with CML despite prolonged treatment with a TKI (Corbin et al. 2011). This field of research is active and focused on identifying the critical pathways of the residual CML leukemia cells.

3.2 Leukemic Stem Cells in AML

In 1994, Lapidot and colleagues published their work characterizing the leukemia initiating capacity of a sub-population of leukemic blasts (Lapidot et al. 1994). Using a SCID xenotransplantation model, they demonstrated that the ability to transplant human AML into primary and secondary recipients was confined to a population of cells defined by the expression of CD34 and the lack of expression of CD38 and markers of lineage commitment. This paper was one of many by the Dick lab examining the potential applications of xenotransplantation to the study of normal and malignant hematopoiesis. In the two decades since the report, the original phenotype has been extended as new antigens capable of enriching for LSC activities have been identified (Table 13.2). These studies have focused primarily on expanding the original $CD34^{+}CD38^{-}$ phenotype.

Beginning in 2008, studies by the Bonnet lab called into question the reliance of the LSC field on the $CD34^{+}CD38^{-}$ phenotype (Taussig et al. 2008, 2010). First, Taussig et al. demonstrated that pre-treatment of unfractionated normal and AML bone marrow cells with antibodies against human CD38 impaired normal and leukemic engraftment in sub-lethally irradiated NOD-SCID recipients (Taussig et al. 2008). Pre-treatment of recipients with an antibody against the interleukin-2 receptor β chain (CD122) partially restored human engraftment through elimination of residual NK cell activity. Transplantation of $CD34^{+}CD38^{-}$ and $CD34^{+}CD38^{+}$ populations from seven AML samples into NSG or NOD/SCID/ß2m^{null}mice pretreated with IVIG or anti-CD122 demonstrated LSC activity in CD34 + CD38+ cells in all cases of AML demonstrating the impact of the model on the cancer stem cell phenotype.

Table 13.2 List of surface antigens reported to identify leukemic stem cell populations in patient samples

Antigen		Model	HSC	LSC	References
CD34	Cell-cell adhesion factor	NOD/SCID	+	+	Lapidot et al. 1994
CD123	IL-3 receptor alpha chain	NOD/SCID	–	+	Jordan et al. 2000
CLL-1	C-type lectin-like molecule 1	NOD/SCID	–	+	Bakker et al. 2004
CD33	Siglec-3	NOD/SCID	+	++	Taussig et al. 2005
CD47	Integrin-associated protein	NOG	+	++	Majeti 2011
CD25	Interleukin 2 receptor, alpha	NOG	–	+	Ishikawa et al. 2007
CD96	T-cell activated increased late protein	Rag2–/–γc–/–	+	++	Hosen et al. 2007
CD32	Fc fragment of IgG, low affinity, IIa receptor	NOG	–	+	Ishikawa et al. 2007
CD45RA	Leukocyte common antigen (LCA)	NOD/SCID and NSG	–	+	Goardon et al. 2011
TIM3	Member of immunoglobulin superfamily	NSG	–	+	Jan et al. 2011

Leukemic blasts from patients with AML associated with mutations in the NPM1 ($NPM1^{mut}$) gene frequently lack surface expression of CD34. Taussig et al. examined the capacity of $CD34^+CD38^-$, $CD34^+CD38^+$, $CD34^-CD38^+$ and the $CD34^-CD38^-$ cells from patients with $NPM1^{mut}$ AML to engraft in their xenotransplantation assay (Taussig et al. 2010). LSC activity was demonstrated for either the $CD34^-CD38^+$, $CD34^-CD38^-$ or both populations. NPM^{mut} cases expressing CD34 demonstrated LSC activity most frequently in the $CD34^+CD38^+$ population. For most cases LSC activity was present in multiple populations demonstrating both inter-patient as well as intra-patient heterogeneity in LSC phenotypes. Using LDA they assessed LSC frequency for the different populations within a single patient. For two patients, the LIC frequency was similar among phenotypically distinct LSC populations. In a third patient, three populations demonstrated similar LSC frequencies while LSCs were less frequent in the fourth, $CD34^-CD38^+$, population.

Sarry et al. (2011) and Eppert et al. (2011) likewise identified inter- as well as intra-patient heterogeneity in LSC surface antigen phenotype, although Eppert et al. (2011) found that LSC frequency was greatest in the $CD34^+CD38^-$population compared to the $CD34^+CD38^+$ population. In all of the above studies, LDA assays confirmed that LSCs make up a rare population of cells in the samples.

Multiple LSC populations within a patient suggest that the LSC pool evolves in response to the addition of other genetic/epigenetic events or occurs as a response to therapy. Cytogenetics, whole genome sequencing and DNA methylation studies have documented changes in the makeup of the malignancy following relapse (Welch et al. 2012; Kroeger et al. 2008). Our group carefully examined the impact

of the patients' clinical course on the LSC phenotype. Using either CD34 and CD38 or CD32 ($CD34^-$ cases) and CD38, we characterized the LSC activity in the four populations defined by these antigens prior to therapy and following relapse using an NSG xenotransplantation model. LDAs of samples obtained prior to therapy and following relapse (Ho et al. 2013) identified a 9–90-fold expansion of functional LSC activity at relapse. There was considerable inter-patient as well as intra-patient heterogeneity in LSC surface antigen phenotypes from diagnostic samples. We demonstrated expansion of LSC activity at relapse to populations lacking LSC activity in the pre-treatment sample. The molecular basis for this evolution and how it relates to the cell of origin is a work in progress.

3.3 Cell of Origin in AML

AML sequencing studies have demonstrated a median of 13 non-synonymous mutations per case (Cancer Genome Atlas Research 2013). How and where these mutations accumulate is an area of great interest. Miyamoto et al. isolated phenotypically distinct populations from patients with AML associated with t(8;21) following attainment of remission (Miyamoto et al. 2000). AML1/ETO transcripts were present in a fraction of stem cells, monocytes, and B-cells isolated from remission samples. Remission marrow cells were plated in methylcellulose and AML1/ETO transcripts were present in erythroid, granulocyte/macrophage, and megakaryocyte colonies. Thus, for AML associated with t(8;21), the cell of origin appears to be a stem cell population capable of giving rise to myeloid cells as well as B-cells. Recently, cell sorting and targeted mutation analysis were combined to investigate which stem or progenitor populations are targeted by specific mutations in AML (Shlush et al. 2014; Corces-Zimmerman et al. 2014; Jan et al. 2012). Following identification of each patient's leukemia-specific profile, Corces-Zimmerman et al. analyzed leukemic cells, T-cells, and HSCs ($CD34^+CD38^-TIM3^-CD99^-$) using targeted amplicon sequencing (Corces-Zimmerman et al. 2014). They confirmed the ability of isolated $CD34^+CD38^-TIM3^-CD99^-$ HSCs to establish multi-lineage engraftment of NSG recipients. Mutations occurring in the HSC populations were termed "Pre-leukemic mutations" and represented a subset of the patient's leukemia specific mutations. Leukemia-specific mutations not present in the HSC populations but present in the purified leukemic $CD99^+$ $TIM3^+$ cells were classified as "late events". Combining this data with that of an earlier study (Jan et al. 2012), they identified 74 mutations in 16 patients with AML; 49 % were preleukemic and 52 % were late events. Mutations overrepresented in the preleukemic group included DNA methylation, chromatin modification, and chromatin topology (IDH2, DNMT3A, ASXL1, and IKZF1) while mutations in FLT3, NPM1 and genes involved in activated signaling were overrepresented in the late event group. $CD34^+$ cells from remission samples identified patient specific pre-leukemic mutations in remission $CD34^+$ progenitors and their progeny. Shlush et al. reported similar findings in a separate cohort of patients (Shlush et al. 2014).

To determine the frequency of commonly occurring mutations in non-leukemic hematopoiesis, (Xie et al. 2014) analyzed data from The Cancer Genome Atlas (TCGA) database. They selected patients with 11 different tumor types but no prior history of a hematologic malignancy or treatment. 2728 individuals were analyzed and a final list of 77 mutations in 58 cases was identified. Sixty-fiour of the events were in 19 genes including DNMT3A, TET2, JAK2, ASXL1, TP53, SF3B1, BCORL1, ASXL2 and SH2B3. Comparing this dataset with the TCGA datasets for myeloproliferative disorders, MDS, AML and chronic lymphocytic leukemia (CLL); DNMT3A, JAK2, TET2, ASXL1, TP53 and SF3B1 were consistently mutated in the TCGA blood dataset and at least two of the other datasets. The authors argued that these mutations were likely to be early events in the initiation of hematologic malignancies. Mutations of IDH1, RUNX1, NRAS, PHF6 were identified in the AML, MPN and MDS datasets but not in the TCGA blood dataset. CEBPA, WT1, PTPN11, KIT, SMC1A, and SMC3 were frequently mutated in the TCGA AML dataset but not in the TCGA blood dataset. These mutations were surmised to be later events.

These studies support a model in which early mutations alter the "landscape" of the HSC pool (Corces-Zimmerman et al. 2014). This altered HSC pool is able to give rise to both myeloid and lymphoid progeny although skewed. Later events in the HSC or its progeny further modify the cell state and activate cell signaling resulting in the ability of cycling malignant progenitor populations to self-renew and completing leukemic transformation (Fig. 13.2). These studies rely on the deconstruction of the tumor and depend on an intact relationship between a cell's surface antigen profile in cancer with that in normal hematopoiesis. With the advent of improved methods to target gene expression to normal cord blood stem cell populations and current xenotransplantation models the tools are in place to prospectively build human leukemias in vivo (Moriya et al. 2012; Chou et al. 2011). IPS technology provides an alternative approach to reverse engineering leukemia (Liu et al. 2014).

3.4 Lessons from Murine Models of Leukemia

Murine models of leukemia allow researchers to take full advantage of the extensive knowledge base of murine normal hematopoiesis and the power of murine genetics. As mutations in human AML are identified, they are modeled in the mouse. A complete review of murine models for leukemia is beyond the scope of this effort. We will highlight two stories where these systems were employed to address key concepts in LSC biology.

Early studies of LSCs in CML and AML suggested that the HSC pool was the primary target for early events with additional events in progenitors resulting in full transformations. Studies in mice have examined the potential of leukemia initiating events to transform HSCs as well as non-self-renewing progenitors. In 2006 Krivtsov et al. initiated acute leukemia in mice by targeting the expression of the

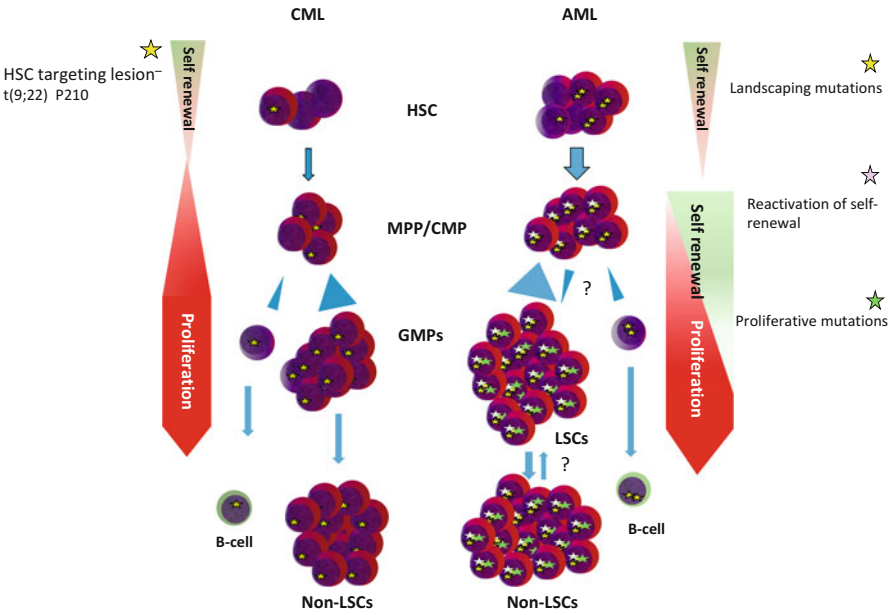

Fig. 13.2 *Model for chronic and acute myeloid leukemia*. (**a**) Chronic myelogenous leukemia is the result of t(9;22) targeting the quiescent HSC. The LSC is a functioning HSC able to give rise to both myeloid and lymphoid progeny with expansion of the myeloid progenitor pools and a granulocytic predominance. Inhibition of the BCR-ABL fusion product restores normal hematopoiesis but does not eradicate the LSC. (**b**) AML. Early genetic events alter the "landscape" of the quiescent HSC pool without completing leukemic transformation. The altered HSC pool is able to give rise to both myeloid and lymphoid progeny although skewed. Later events in the progeny activate self-renewal and cell signaling resulting in the ability of cycling malignant progenitor populations to self-renew and completing the leukemic transformation. As the disease progresses, more committed progenitor populations acquire the potential for self-renewal. It has yet to be determined if these states are inter-exchangeable in human disease as demonstrated by Nolan et al. in a murine model

MLL-AF9 fusion product to GMPs (Krivtsov et al. 2006). They demonstrated that the LSCs possess an immunophenotype and gene expression profile similar to that of normal GMPs. A subset of genes (363) in the MLL-AF9 LSC signature overlap with the expression profile of normal murine HSCs and the expression of profiles of human MLL associated AML. Subsequent studies have employed this model to refine the critical events in MLL mediated leukemia as well as identify novel therapeutics (Krivtsov et al. 2013; Wang et al. 2010; Hanoun et al. 2014; Miller et al. 2013). A similar capacity to activate a self-renewal program in progenitor populations has been has been demonstrated for the MLL-ENL and MOZ-TIF2 fusion products (Huntly et al. 2004; Cozzio et al. 2003). This capacity is not shared among all driver mutations BCR-ABL, Flt3 ITD mutations as well as the HOXA9-MEIS1 fusion product are not capable of fully transforming normal progenitors. Interestingly, MLL-AF9, when driven under the endogenous MLL promoter is unable to transform GMP populations.

Data from human studies supports the presence of multiple distinct LSC populations in patients with AML. Gibbs et al. (2012) applied Cytof technology to characterize LSCs populations in a murine model for AML driven by the HoxA9-Meis1 fusion. Three distinct LSC populations capable recapitulating the original immunophenotype on transplantation were identified, $Lin^{-}kit^{+}$, $Gr1^{+}kit^{+}$ and $Lym^{+}kit^{+}$. Detailed Cytof analyses of recipient marrow following transplantation of these independent LSC populations demonstrated shared signaling networks. The authors concluded that their data was not consistent with a unidirectional process of differentiation and that stemness may reflect a cellular state that exists independently of surface antigen definition.

3.5 Stem Cells in Myelodysplastic Syndromes

The myelodysplastic syndromes are a heterogeneous group of diseases characterized by bone marrow failure and a variable risk for transformation to acute leukemia. Intermediate and high risk MDS represent pre-leukemic states and are treated as such. MDS is characterized by recurrent cytogenetic and molecular abnormalities and sequencing efforts have identified overlap of the mutational spectrum in MDS with AML (Bejar et al. 2011). Recognized as a stem cell disorder early on (Raskind et al. 1984), clonal involvement of the HSC pool and its progeny is supported by studies in which HSC and early progenitors demonstrate the presence of patient specific genetic events (Nilsson et al. 2002; Tehranchi et al. 2010; Will et al. 2012). HSC and progenitor populations from primary patient samples demonstrate changes in the size of the HSC, CMP, CMP and MEP pools (Will et al. 2012). Studies evaluating the impact of therapy on the HSC pool in patients with MDS have established that despite clinical responses to lenalidomide or 5-azacytidine, including remission, the malignant HSC pool remains untargeted and serves as a reservoir for relapse (Will et al. 2012; Craddock et al. 2013; Tehranchi et al. 2010). Until recently, functional assessment of clonal HSCs and progenitors from patient samples has been hampered due to difficulties in achieving sustained engraftment of clonal hematopoiesis immune deficient mice (Nilsson et al. 2000, 2002). Two recent reports demonstrate sustained clonal engraftment of recipient mice following co-transplantation of sorted hematopoietic cells with bone marrow stromal cells via intrafemoral injection (Muguruma et al. 2011; Kerbauy et al. 2004).

With the identification of disease driving genetic events in MDS, the development of novel murine models for MDS that mirror human disease is also likely to help define the role of fully transformed stem cell populations in MDS. Early murine models for MDS were mainly limited to the less common CMML category while recent models demonstrate a phenotype more representative of the breadth of MDS (Barlow et al. 2010; Moran-Crusio et al. 2011). A recent model in which expression of the Dicer gene was targeted in bone marrow osteoblasts highlighted the role of the bone marrow microenvironment in the pathophysiology of MDS (Raaijmakers et al. 2010). These models will allow the investigation of the functional cancer stem cell populations in MDS.

3.6 Molecular Characterization of LSCs

While there have been a large number of reports applying gene expression analysis to characterize samples from large cohorts of patients with AML, only a few have studied the malignant stem cell populations (Ishikawa et al. 2007; Guzman et al. 2001b; Goardon et al. 2011; Gentles et al. 2010; Majeti et al. 2009; Eppert et al. 2011) . Guzman et al (2001b) examined the expression levels of 1400 genes related to cancer and apoptosis in leukemic $CD34^{+}CD38^{-}$ cells from primary patient samples and demonstrated aberrant expression of DAPK and IRF-1. Studies that followed applied microarray technology to compare leukemic $CD34^{+}CD38^{-}$ cells to leukemic $CD34^{+}CD38^{+}$ cells (Ishikawa et al. 2007), leukemic $CD123^{+}CD34^{+}$ $CD38^{low}$ $Lineage^{-}$ cells to normal HSCs (Majeti et al. 2009; Gentles et al. 2010) and finally functionally defined LSC and normal HSC populations to leukemic populations lacking LSC activity (Eppert et al. 2011). Two of these signatures were shown to have prognostic significance (Eppert et al. 2011; Gentles et al. 2010). These differing approaches have yielded varying results with limited overlap but form a database for future queries. The LSC signature generated by Eppert et al. was enriched for an HSC signature. They also compared their LSC signature with data sets derived from embryonic stem cell as well as hematopoietic stem and progenitor populations. Their LSC signature was positively correlated with published HSC signatures and negatively correlated with more differentiated cell signatures. Majeti et al. compared normal HSCs to leukemic $CD123^{+}CD34^{+}$ $CD38^{low}$ $Lineage^{-}$ cells and identified the *Adherens junction, Ribosome, Regulation of actin cytoskeleton, Tight junction and Focal adhesion* pathways (KEGG) as the top five dysregulated pathways in leukemic progenitors. With the advent of new platforms to characterize miRNA levels as well as DNA methylation changes, integration of these datasets with functional data will be critical.

3.7 Therapeutic Targeting of LSCs in Myeloid Malignancies

Two decades have passed since publication of the study by Lapidot et al. (1994) that launched the stem cell model for AML. While there is agreement in the need to identify agents capable of eradicating the malignant stem cell population; increasing recognition of the heterogeneity of the LSC pool in and between patients complicates the design and implementation of LSC targeted therapies. The study of normal stem cell biology has identified a number of shared pathways that are responsible for self-renewal including sonic hedgehog, Wnt, Notch, and BMI1. These pathways are known to be necessary for malignant stem cell function in multiple tumor types. Efforts to target these pathways are in different phases of translation to the clinic.

A second approach to targeting cancer stem cells is to identify surface proteins present on cancer stem cell populations that are lacking or expressed at a lower level

on normal stem cell and progenitor populations (Table 13.2). Anti-CD33 therapy has been in the clinical arena for over a decade; while there is a benefit to a small subset of patients; this approach overall has been disappointing (Burnett et al. 2011). Similar early phase clinical efforts for CD123, CLL1 are ongoing as are pre-clinical studies targeting CD44, CD47, TIM-3 and IL1RAP (Majeti 2011; Askmyr et al. 2013; Kikushige and Miyamoto 2013). In addition to standard monoclonal antibody therapy, Bi-specific T-cell engagers (BiTEs) and CAR-T efforts using C33, CD123 and LeY antigens are in the pre-clinical and early clinical stages (Aigner et al. 2013; Ritchie et al. 2013; Gill et al. 2014; Dutour et al. 2012). A major limitation of the LSC antigen targeting approach is that none of the currently published LSC antigens appear to be both present on all of the LSC populations in a patient while lacking on normal HSCs and/or progenitors.

A third approach is to target unique cancer stem cell dependencies. The development of mutation specific therapies is one such approach although most mutations are limited to a small fraction of patients except for NPM1 and FLT3 mutations. An early study demonstrated constitutive NF Kappaβ activity in LSCs as compared to normal HSCs (Guzman et al. 2001a). This has been confirmed and a recent study demonstrated that NF Kappaβ activity was common to murine and human LSC populations and associated with expansion of the LSC pool (Kagoya et al. 2014). An ongoing Children's Oncology Group trial is investigating the benefits of adding bortezomib to standard induction therapy. In 2005 Guzman et al. published a follow-up study in which they characterized the ability of a natural product, parthenolide, to selectively eradicate LSC populations (Guzman et al. 2005). Initially selected for its ability to inhibit NF Kappaβ signaling (Hehner et al. 1999), subsequent studies have identified alternative activities of this class of compounds including redox balance, heat shock protein response, proteasome signaling and glycolysis (Pei and Jordan 2012; Pei et al. 2013). Subsequent studies have confirmed the utility of this class of compounds in targeting the above pathways and identified other molecules with similar effects or that synergize with parthenolide (Lagadinou et al. 2013; Dai et al. 2010; Hassane et al. 2008, 2010).

4 The Role of Malignant Stem Cells in B-cell Malignancies

4.1 Normal B-cell Development

In the current model for B-cell development, multi-potent progenitors undergo a cell fate decision giving rise to a multipotent lymphoid progenitor that then give rise to either B-cells or T-cells with the early pro-B-cell the first stage of B-cell development (Fig. 13.1). Early B-cell development occurs in the bone marrow followed by exodus to the lymph organs. Following selection and maturation in the germinal centers of primary and secondary lymph organs, B-cells return to the bone marrow. Developmental stage can be identified using the expression of specific

surface antigens and the rearrangement status of Ig H and L chains. $CD34^{+}CD10^{+}CD19^{-}$ define the common lymphoid progenitors while pro-B-cells are defined as $CD34^{+}CD10^{+}CD19^{+}$ and pre-B-cells are $CD34^{-}CD10^{+}CD19^{+}$. Rearrangement of the VDJ H chain locus is characteristic of pro-B-cells while expression of a pre-BCR, composed of IgH chains and surrogate L chains marks the pre-B-cell population (Hystad et al. 2007). Memory B-cells, plasmablasts and mature plasma cells are similarly defined by their surface antigen expression profile (Memory B-cell: $CD27^{+}CD20^{+}CD45^{++}IL6R^{-}CD138^{-}CD38^{dim}$; Plasmablast: $CD20^{-}CD38^{++}CD45^{++}IL6R^{++}CD138^{-}$ and Mature plasma cells: $CD20^{-}CD38^{++}CD45^{dim}IL6R^{+}CD138^{++}$).

Cells of the myeloid lineage including erythrocytes, megakaryocytes, and granulocytes have well defined and frequently quite short half-lives while a subset of mature B-cell populations are long lived. This is the basis for immunologic memory allowing for a more rapid antigen specific response following re-exposure by rapid expansion of memory B-cells. In 2006, Luckey et al. (2006) analyzed the expression profiles of naïve, effector and memory T-cells as well as naïve, germinal center, memory B-cells and plasma cells. Transcripts augmented in memory cell populations compared to naïve and effector cell populations were enriched in HSCs and lost following commitment. Likewise, transcripts down regulated in memory cell populations were down regulated in HSCs and increased with differentiation.

4.2 The Role of Leukemic Stem Cells in B-cell ALL

B-cell Acute Lymphoblastic leukemia (B-ALL) arises in an early B-cell progenitor (Teitell and Pandolfi 2009) from the accumulation of genetic events by hematopoietic stem cells and/or progenitor cells. Early immunoglobulin rearrangement studies demonstrated that a quarter of early B-ALL cases contain more than two IgH rearrangements. Sequential analyses of a few patients found that the patient specific pattern of IgH gene rearrangement may change over the course of the disease (Wright et al. 1987). TEL-AML1 B-ALL is a rare disease and cases of in-utero transfer of a pre-leukemic clone between twins have been reported. When one twin presents with B-ALL, the other twin may harbor the preleukemic clone. Hong et al. demonstrated that $CD34^{+}CD38^{-}CD19^{+}$ leukemic cells from four patients with TEL-AML1 B-ALL were capable of transplanting the leukemia in mice (Ma et al. 1998). The peripheral blood of the healthy twin of one patient contained found very rare circulating TEL-AML1 positive $CD34^{+}CD38^{-}CD19^{+}$ pre-leukemic cells. Castor et al. (2005) examined the involvement of $CD34^{+}CD38^{-}CD19^{-}$ and $CD34^{+}CD38^{-}CD19^{+}$ cells from children and adults with B-ALL for presence of the TEL-AML1, P190 BCR-ABL and P210 BCR-ABL translocations. TEL-AML1 and p190BCR-ABL involved the B-cell progenitor population but not the $CD34^{+}CD38^{-}CD19^{-}$ HSC pool while p210BCR-ABL cases involved both populations. Xenotransplantation studies revealed that only the $CD34^{+}CD38^{-}CD19^{+}$ cells from TEL-AML1, P190 BCR-ABL and p210BCR-ABL cases gave rise to B-ALL

upon transplantation. $CD34^+CD38^-CD19^-$ cells gave rise to normal reconstitution regardless of the type of B-ALL. This suggests that for these pediatric (TEL-AML1 and p190BCR-ABL) and adult (p210BCR-ABL) B-ALL, the cell of origin may differ but the functional LSC has a conserved phenotype, that of a committed B-cell progenitor. Cobaleda et al. (2000) isolated leukemic cells from B-ALL samples associated with t(9;22). In contrast to the study by Castor et al. (2005), only CD34 + CD38- cells were capable of engrafting NOD/SCID mice. Cox et al. (2004) employed a series of in vitro assays as well as xenotransplantation to characterize the surface antigen profile of B-ALL leukemia initiating cells. LSC activity was restricted to the $CD34^+CD10^-$ and $CD34^-CD19^-$ populations. This study did not include B-ALL associated with a t(9;22) or t(4;11).

The above studies relied on NOD/SCID assay and studies in AML have shown the potential impact of this assay on the phenotypes of LSC population(s) identified. Rau et al. (2014) undertook an extensive study of primary ALL samples using an NSG model with intrafemoral transplantation. The patients represented three distinct ALL risk groups. Despite using a number of surface antigens, they were unable to enrich for LSC activity. All populations demonstrated similar engraftment frequencies as well as kinetics of engraftment. LDA of the sorted populations demonstrated similar frequencies for LSC activity regardless of surface antigen phenotype. Their data was consistent with the lack of a hierarchy in acute B lymphoblastic leukemia and suggest a non-hierarchical model to account for tumor heterogeneity.

Efforts are underway to improve human B-ALL LSC models using cord blood stem cell targeting and xenotransplantation. Barabe et al. (2007) targeted the expression of the MLL-ENL and MLL AF9 fusion transcripts in primary cord blood progenitor cells. Recipient mice developed both B-ALL and AML that was transferrable into secondary recipients. Retroviral insertion analyses and IgH locus analyses demonstrated that the B-ALL recipients contained differing rearrangements of the IgH locus. 40 % of the cases were consistent with transformation of an early hematopoietic progenitor. Using serial transplantation to model disease progression, they showed that the contribution of clones with unarranged IgH loci diminished with passage while alternative LSC populations arising from more mature B-cell progenitors maintained the disease.

4.3 Myeloma Stem Cells

Multiple myeloma (MM) belongs to a spectrum of diseases that includes monoclonal gammopathy of undetermined significance (MGUS), smoldering myeloma and symptomatic myeloma. MGUS is present in approximately 3 % of the general population 50 years of age and older with a risk of transformation to multiple myeloma of 1 % per year. In 2009, it was demonstrated that in up to 75 % of myeloma patients a detectable M-protein was identifiable 8 or more years prior to diagnosis (Weiss et al. 2009). This is consistent with a clonal process maintained by a population(s)

of cells that are long lived. Immunoglobulin sequencing studies in patients with MM demonstrates the presence of extensive hypermutation without intraclonal variation consistent with the development of the malignancy at the post-germinal center B-cell.

Myeloma was one of the cancer types in which tumor heterogeneity was initially assessed. In 1968, Bergsagel and Valeriote characterized the capacity of a murine plasma cell line to initiate a malignancy in recipient syngeneic mice (Bergsagel and Valeriote 1968). 3×10^4 malignant plasma cells were required to form at least one colony in the spleen of recipient mice. They used this model to show that most of the plasma cells were cycling and sensitive to vinblastine, a cell cycle dependent chemotherapeutic agent. In 1977, these findings were reproduced using human myeloma cells. More recent efforts have employed two different approaches to address heterogeneity in plasma cell tumors; NOD/SCID and NSG xenotransplantation models such as those noted above or models in which human (SCID-hu) or rabbit (SCID-rab) bone fragments are implanted directly into immunocompromised mice followed by injection of the myeloma cells into the implant.

In 1998, (Yaccoby et al. 1998) employed a SCID-Hu xenotransplantation model for studying primary MM samples. $CD38^{++}CD45^{-}$plasma cells engrafted the implanted human bone whereas the plasma cell depleted cells did not. In normal B-cell development, terminal plasma cell differentiation is characterized by loss of CD45 and increasing CD38 expression. Hosen et al. reported CFU activity as well sustained engraftment in the SCID-rab model for $CD138^{-}CD19^{-}CD38^{++}$ cells (Hosen et al. 2012). $CD19^{+}$ B-cells lacked CFU activity and engraftment potenital. $CD138^{+}$ cells engrafted in a subset of the cases.

Matsui and colleagues used magnetic bead enrichment/depletion approaches to obtain $CD138^{+}CD34^{-}$ and $CD138^{-}CD34^{-}$ bone marrow cells from 24 MM patients. $CD138^{+}CD34^{-}$ cells were unable to form colonies in vitro while the $CD138^{-}CD34^{-}$ cells generated colonies of morphologically mature plasma cells expressing CD138. Engraftment of NOD/SCID mice by myeloma was observed only with $CD138^{-}CD34^{-}$ cells which gave rise to $CD138^{+}$ cells in the recipient mice. MM samples depleted of CD19, CD45 and CD20 cells lacked colony forming potential consistent with a memory B-cell-like progenitor. They refined the surface antigen profile of myeloma propagating populations in a follow-up report, $CD138^{-}CD27^{+}CD19^{+}$, and demonstrated that this population was resistant to lenalidimide, bortezomib and cyclophosphamide; agents commonly used to treat patients with MM (Matsui et al. 2004; Matsui et al. 2008).

In 2013, Chaidos and colleagues addressed the disagreement as to the surface antigen phenotype of MM cells responsible for tumor maintenance (Chaidos et al. 2013). They extensively characterized the surface antigen profiles of MM blood and marrow samples coupled with IgH CDR3 characterization. They found that the MM clone did not include pre-germinal center B-cells but was comprised of a mixture of mature $CD19^{+}$ B-cells (resting memory B-cells), plasmablasts, and $CD138^{low}$ and $CD138^{+}$ plasma cells. They also identified a $CD138^{-}$ population they termed the pre-plasma cell (pre-PC). They sorted and transplanted $CD19^{+}$ B-cells, Pre-PCs and PCs from MM patients into sub lethally irradiated NSG mice. Recipients of $CD138^{+}$

PCs displayed BM engraftment in 9 of 12 cases. Negatively enriched Pre-PCs also demonstrated engraftment while none of the mice receiving $CD19^+$ cells showed evidence of engraftment. They proposed a model in which myeloma-stem cell activity was confined to two interconvertible populations of MM cells distinguishable only by the level of expression of CD138. It is unclear to what degree the heterogeneous results reported relate to clinical features of the samples studied, the approach to isolating populations (negative selection vs positive selection) or the different models employed for xenotransplantation assays. Alternatives to a reliance on surface antigen profiling have employed sorting based on ALDH expression or Hoechst 33,342 staining (Matsui et al. 2004). Additional studies will be necessary to further clarify the exact nature of the myeloma initiating cell using a uniform and standardized set of isolation approaches and xenotransplantation model.

4.4 Targeting Malignant Stem Cells in B-cell ALL and Myeloma

Similar to other cancers, pathways regulating normal stem cell self-renewal have been examined in B-ALL and MM. The sHH pathway is active in MM stem cells and early pre-clinical efforts have demonstrated some efficacy (Agarwal et al. 2014; Peacock et al. 2007).

CD19 is expressed on the cell of origin and the LSC for many cases of B-cell ALL and its expression is carried through late into B-cell development (see above). There is general excitement about several novel approaches to targeting CD19 in B-ALL and other B-cell malignancies. Monoclonal antibodies against CD19 have been conjugated to antineoplastic agents as well as to a single-chain variable region capable of binding to the CD3 T-cell receptor. These agents have shown activity in early phase trials and are now being tested in the upfront setting. Likewise, CAR-T-cells targeting CD19 have had success in relapsed B-ALL (Maude et al. 2014; Davila et al. 2014). These efforts have been expanded to most B-cell malignancies. The observation that MM propagating cells express CD20 led to a small clinical trial employing Rituximab, a monoclonal antibody against CD20 (Moreau et al. 2007). This trial demonstrated little efficacy for this approach in patients and this approach has not moved forward. Antibodies targeting CD138, present on some MM stem cell phenotypes but not others, has been studied and is currently undergoing phase 3 testing.

5 Conclusion and Future Directions

In 2011, Hanahan and Weinberg updated their treatise on the hallmarks of cancer which include sustaining proliferative signaling, evading growth suppressors, resisting cell death and enabling replicative immortality (self-renewal) (Hanahan and

Weinberg 2011). These properties are shared by normal cells during normal development and homeostasis where they are compartmentalized and tightly regulated. Although initial studies in AML and CML demonstrated an overlap of the cancer stem cell phenotype with that of the normal HSC; subsequent studies have shown that in cancer the capacity for self-renewal can expand to less quiescent progenitor populations. As the disease progresses, the cancer stem cell phenotypes in AML, MM and ALL resemble late progenitors as "stemness" moves further out into the hierarchy. It will be critical to define the cancer specific mechanisms driving this expansion as targeting these pathways may restore control of self-renewal without restricting self-renewal in normal populations. Recently, constitutive activation of the nuclear factor-kappa B (NF-κB) pathway was shown to expand functional LSC activity as assessed by LDA (Kagoya et al. 2014). As additional agents capable of targeting key pathways involved in self-renewal become available, studying their impact on all compartments in cancer and normal tissue will be critical.

Whole genome sequencing studies have outlined the genetic space for most hematologic malignancies. Interestingly, studies of normal appearing hematopoietic stem cells in the diagnosis and remission samples from patients with AML have identified cells retaining a capacity for multi-lineage differentiation with disease specific mutations. These may represent residual pre-leukemic stem cells with overlap of these mutations with those identified in peripheral blood and marrow samples from individuals without a hematologic malignancy. The frequency and nature of mutations in individuals without a hematologic malignancy is tightly associated with aging as are the diagnoses of AML, MDS and Myeloma. These findings will need to be validated in aged healthy individuals with follow-up analyses to ensure they affect a long-lived population. We will require a greater understanding of how these mutations alter the landscape of the stem cell pool prior to accumulating additional genetic events. A frequent statement in reviews and articles on cancer stem cells is that CSCs represent a reservoir for relapse hence the interest in identifying and phenotyping these populations. How to effectively target a pre-leukemic stem cell pool that may be indistinguishable from normal HSCs will likely serve as a task for the next decade. As demonstrated so well by chronic phase CML, it may not be necessary to eradicate pre-leukemic HSCs as long as there are effective therapies for their progeny.

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Chapter 14
Leukemic Stem Cells in Acute Lymphoblastic Leukemia

Ugo Testa

Abstract Acute lymphoblastic leukemia (ALL) is observed in both children and adults, with >60 % of cases occurring at age <20 years (peak incidence at ages between 2 and 5 years). The survival of pediatric ALL, particularly of children ALLs is around 90 %, while the prognosis of adults and infant ALLs is poor. According to their differentiation features, ALLs are distinguished in B-ALLs and T-ALLs. T-ALLs are less frequent than B-ALLs, accounting for 25 % of adult T-ALLs and 10–15 % of pediatric ALLs. The nature and the frequencies of the stem cells or leukemia-initiating cells in ALLs were intensively investigated during last years. The ensemble of these studies carried out on the characterization of leukemic stem cells in B-ALLs indicate that the putative stem cells responsible for initiating and maintaining B-ALLs are not a fixed cellular identity (i.e., cells with $CD34^+CD19^+$ or $CD34^+CD19^-$ or $CD34^-$ have been shown to possess leukemia-initiating capacity), but themselves evolve both in their genotype and phenotype. This conclusion was strongly supported by studies carried out in twins: basically, these studies have shown that the ALL-specific fusion events occur in utero during embryonic/fetal development, generating a preleukemic clone, clinically silent; the preleukemic clone may progress to full leukemia development through the acquisition of new genetic abnormalities, such as point mutations, deletions and/or duplications. In addition to these findings, another very important contribution derived from the study of B-ALL leukemia-initiating cells is that these cells are not only phenotypically heterogeneous, but also genotypically heterogeneous: their heterogeneity reflect the heterogeneity of the bulk tumor cells. Furthermore, relapsing B-ALLs are issued from leukemic stem cell populations, representing a major or minor clone at presentation.

Keywords Hematopoiesis • Hematopoietic stem cells • Leukemic stem cells • Acute lymphoblastic leukemias

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1 Normal Hematopoiesis

Hematopoiesis represents a complex multistep differentiation system initiated by undifferentiated stem cells (hematopoietic stem cells, HSCs) undergoing a process of self-maintenance and of cell differentiation through the generation of a series of hematopoietic progenitor cells (HPCs), multipotent and unipotent. The homeostasis of blood production is carefully controlled through regulation of self-renewal, lineage specification, differentiation and maturation. Therefore, the two essential processes regulating hematopoiesis are mainly dependent on the capacity of HSCs to both self-renew and differentiate. The studies carried out on both murine and human HSCs and HPCs during the l ast decades have led to elucidate in part the mechanisms and the cellular steps through which HSCs progressively differentiate.

To assay human hematopoietic stem and progenitor cells, immunodeficient animals, such as nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice, are currently used. Cells endowed with the capacity of repopulating at long-term the hematopoietic system of these immunodeficient mice, as well as of secondary animal recipients, are thought to be HSCs. The NOD/SCID repopulating assay provided evidence that human HSCs are present in the $CD34^+CD38^-$ fraction of human hematopoietic cells. Using NOD/SCID mice strains with enhanced immunosuppression as recipients, it was shown that also the $CD34^+/CD38^+$ cell fraction possesses some repopulating activity. However, $CD34^+/CD38^+$ cells possess only a short-term SCID-repopulating activity, while the long-term repopulating activity is limited to the $CD34^+/CD38^-$ cell population (Hogan et al. 2002). It is important to mention that several studies have characterized a rare SCID-repopulating population observed at the level of $CD34^-Lin^-$ cells: these cells, like $CD34^+/CD38^-$ cells possess a long-term repopulating capacity. Importantly, $CD34^-$ HSCs are able to generate in vivo $CD34^+$ HSCs (Kimura et al. 2010).

According to their capacity of repopulating hematopoiesis, the hematopoietic stem cell pool can be subdivided into three groups: short-term HSCs, capable of generating clones of differentiating cells for only 4–6 weeks; intermediate-term HSCs, capable of sustaining a differentiating cell progeny for 6–8 months before becoming extinct; long-term HSCs, capable of maintaining hematopoiesis indefinitely (Benviste et al. 2010).

The classical model, the *clonal succession model*, of hematopoietic hierarchy implies that all mature cells of the peripheral blood are the progeny of a single long-term hematopoietic stem cell. An alternative model, the *clonal diversity model*, proposes that distinct hematopoietic stem cell types are capable to contribute to the formation of all lineages, but are programmed to do so in a highly biased fashion, in part related to microenvironmental stimulations. Recent studies provide support to the second model. These observations are compatible with the view that the hematopoietic system is maintained by a continuum of hematopoietic stem cell subtypes, rather than a functional uniform stem cell pool.

All blood elements are generated at the level of the sites where hematopoietic stem cells reside (i.e., in the fetal liver during fetal life and in the bone marrow

during postnatal life), with the exception of T-cells that are generated at the level of the thymus from lymphoid progenitors derived from HSCs and migrating in this site. The first-cellular elements generated by the differentiation of HSCs consist of progenitors of more restricted differentiation capacity, until to generate unipotent progenitors. Collectively, all this process is known as lineage commitment or cell fate decision. Following a model proposed for many decades, the hematopoietic stem cell first originates a common myeloid progenitor (CMP), able to generate all myeloid lineages (granulocytes, monocytes, dendritic cells, erythrocytes and megakaryocytes) and a common lymphoid progenitor (CLP), able to generate all lymphoid elements, B, T and NK lymphocytes. Alternatively to this view, more recently it was proposed an alternative myeloid-based model, postulating that HSCs first diverge into a CMP and a common lymphoid-myeloid progenitor (CLMP). The CMPL in turn generates T and B-cell progenitors through a bipotential myeloid-T progenitor and myeloid-B progenitor stage, respectively (Kawamoto et al. 2010).

This last model received support from recent studies based on the characterization of early human hemato-lymphopoietic progenitors. Thus, according to the actual view during normal human hematopoiesis two types of multipotent progenitors are generated: a CMP, isolated from bone marrow as a $CD34^+CD38^+IL\text{-}3R\alpha^+CD45RA^-$ cell, unable to generate every type of lymphoid cells, and capable of generating all myeloid elements through an intermediate differentiative pathway involving the generation of bipotent G/Mo and Mk/E progenitors; a CLMP which can be identified as a $CD34^+CD38^-CD45RA^+Thy\text{-}1^{neg\text{-}low}$ cell, capable of generating in vitro and in vivo all lymphoid elements (T, B and NK lymphocytes) and some myeloid elements such as monocytes/macrophages and dendritic cells, but not erythroid, megakaryocytic or granulocytic elements (Doulatov et al. 2010).

More recently, the fractionation of human cord blood and bone marrow $CD34^+$ cells into $CD133^+$ and $CD133^-$ subfractions allowed to propose a revised dichotomy model, where the HSCs are able to generate two types of multipotent progenitors, a CMP, here defined as a common erythro-myeloid progenitor (EMP) capable of generating erythroid cells, megakaryocytes, basophilic and eosinophilic granulocytes, and a LMPP, capable of generating lymphoid elements, dendritic cells and granulocytes (Gorgens et al. 2013).

The large majority of HSCs are $CD34^+$. Many studies have shown that in various hematopoietic tissues (bone marrow, cord blood, peripheral blood, and mobilized peripheral blood), the $CD34^+CD38^-$ cell fraction is enriched in early multipotent progenitors, while the $CD34^+CD38^+$ fraction is enriched in committed progenitors. Other membrane markers expressed on $CD34^+CD38^-$ cells further enrich for HSCs, such as CD90. Furthermore, it was shown that CD45RA was expressed on HPCs, but not on HSCs. Therefore, the selection of $CD34^+CD38^-CD90^+CD45RA^-$ allowed to considerably enrich in HSCs. CD49f was shown to be a more reliable marker for human HSCs than CD90 in that virtually all HSCs were shown to be $CD49f^+$ (Notta et al. 2011a). The combination of some membrane markers allows also to enrich for selected types of HPCs: thus, $CD34^+CD38^-CD90^{low/-}CD45RA^+CD135^+$ (Doulatov et al. 2010) or $CD34^+CD38^-CD45RA^+CD10^-$ (Goardon et al. 2011) allows to identify and to enrich for progenitors with LMPP features. Gorgens et al. (2013) have

identified different subsets of HPCs according to the membrane antigen phenotype: (1) the majority of erythro-megakaryocytic progenitors are observed among $CD34^+CD133^-$ cells; furthermore, the eosinophilic and basophilic granulocytic progenitors are also observed among $CD34^+CD133^-$ cells (therefore, the large majority of MEPs are observed among $CD34^+CD133^-$ cells); (2) MPPs, LMPPs, MPLs and GMPs reside at the level of the $CD34^+CD133^+CD138^{-/low}CD45RA^-$; LMPPs as $CD34^+CD133^+CD38^{-/low}CD45RA^+CD10^-$; GMPs as $CD34^+CD133^+CD38^+CD45RA^+CD10^-CD7^-$. These progresses at the level of the identification of various subtypes of hematopoietic stem/progenitor cells were of fundamental importance for the study of the microRNAs at the level of the various types of HPCs.

In addition to $CD34^+$ HSCs, the human HSC hierarchy contains a rare $CD34^-$ population, able to repopulate hematopoiesis into immunodeficient mice. These cells were characterized as $CD34^-CD38^-CD93^+$ cells, have characteristics of HSCs and can be placed in the HSC hierarchy above $CD34^+$ HSCs. A remarkable property of these cells is that consists in an active NOTCH signaling (Anjos-Afonso et al. 2013).

HSCs exhibit several biological properties different from other hematopoietic cells, including HPCs. Thus, given their consistent longevity, HSCs are exposed during their long life-time to various stress stimuli, including reactive oxygen species, nutrient fluctuation and DNA damage and then they possess particular mechanisms that govern their integrity through the unfolded protein response pathway (van Galen et al. 2014). Another remarkable property of HSCs is their requirement for a highly controlled rate of protein synthesis, lower than that observed in other hematopoietic cells (Signer et al. 2014). HSCs are located at the level of peculiar tissual areas known as stem cell niches, essential for maintaining and regulating these cells: these niches are perivascular and are composed by mesenchymal stromal cells and endothelial cells and are usually located near trabecular bone (Morrison and Scadden 2014). HSC populations are heterogeneous, as shown by the intrinsically determined heterogeneity in differentiation potentiality of long-term HSCs and neutrophil-restricted human HSC with rapid, but transient repopulating activities (Miller et al. 2013). A striking example of HSC heterogeneity was obtained in a recent study showing that the expression of the glycoprotein von Willebrand Factor (vWF) identifies a HSC subset that is primed for megakaryocytic production in response to thrombopoietin; vWF^+ $HSCs^+$ are able through their self-renewal to generate both vWF^+ and vWF^- daughter HSCs (Sanjan-Pla et al. 2013).

Among the various factors, low oxygen tension (hypoxia) plays a key role in maintaining undifferentiated states of hematopoietic stem cells: gradients of oxygen between 1 % in hypoxic niche and 6 % in the sinusoidal cavity exist in the human bone marrow. Therefore, HSCs and primitive HPCs exhibit a hypoxic profile and harbor metabolic properties of hypoxic cells, such as enhanced anaerobic glycolysis and reduced flux through the Krebs cycle. Therefore, the hypoxic microenvironment plays a major role in metabolic reprogramming of HSCs and in regulation of their function. Cellular responses to hypoxia are mediated by hypoxia-inducible factors (HIFs), regulating gene expression in a way that permits to the cells an adaption to the hypoxic condition. Using a HIF-1-mediated modification of the gene

expression program, hematopoietic stem cells adapt to the hypoxic microenvironment within stem cell niches by utilizing glycolysis instead of mitochondrial phosphorylation (Simsek et al. 2010); HIF-1α deficiency at the level of the hematopoietic stem cell compartment causes an increased cell cycling rate and progressive loss of long-term repopulating activity (Takubo et al. 2010). HIF-2α is less expressed than HIF-1α in HSCs, but both HIF-1α and HIF-2α transcripts are more abundantly expressed in HSCs than in HPCs. In contrast to the findings observed in studies of HIF-1α gene knockout, acute (inducible) or constitutive conditional deletion of HIF-2α specifically at the level of the hematopoietic system, had no impact on HSC survival and self-renewal. Therefore, these observations suggest that HIF-1α and HIF-2α may have different functions in mouse HSCs (Guitart et al. 2013). In contrast to the findings observed in murine HSCs, HIF-2α knockdown in human cord blood HSCs and early HPCs showed an inhibitory effect on their proliferation, a reduced ability to form erythroid colonies in vitro and an impaired ability to reconstitute hematopoiesis in vivo, due to enhanced production of ROS and increased endoplasmic reticulum stress (Rouault-Pierre et al. 2013).

Lymphocyte development implies the regulated production of B- and T-lymphocytes from HSCs through a series of progenitors exhibiting a progressively restricted differentiation capacity. The first of these differentiative steps involves the generation of multipotential progenitors, termed multipotent progenitors (MPPs), which have still the capacity to generate all hematopoietic lineages, but have lost the self-renewal property. The up-regulation of the membrane receptor on these cells characterizes the lymphoid commitment; these cells have been described as LMPPs. The subsequent step of differentiation is characterized by the loss of the myeloid potential and by the up-regulation of the IL-7R; these progenitors are known as Common Lymphoid Progenitors (CLPs). CLPs have the capacity to generate all types of lymphoid elements, including B-, T- and NK-lymphocytes, as well as lymphoid dendritic cells. The commitment to the B-cell or T-cell lineages following migration to the thymus implies the loss of the potential for other lineages. A number of transcription factors important for early B-lymphoid commitment, including PU.1, Ikaros, Pax5 Hhex and E2A, promote B-cell differentiation. On the other hand, following migration to the thymus, a distinct group of transcription factors, including E2A, Lyl1, TCF1, PU.1, Myb, GATA-3, C/EBPalpha and Mef2c, promotes the early steps of T-cell differentiation and the expansion of early thymocyte progenitors (ETPs).

The first early stages of human T-cell differentiation have been in part delineated, providing a model of T-cell differentiation which implies: (i) first the migration of lymphoid stem/progenitor cells; (ii) the initial differentiation of these cells reaching a differentiation stage (DN1) at whose level are still multipotent (i.e., they can generate T and B-lymphocytes, macrophages and dendritic cells); (iii) during the subsequent stage of differentiation (DN2) the thymic progenitor cells become located in thymic niches where, under the effect of Notch1 and IL-7, undergo the first steps of T-cell commitment; (iv) under the effect of thymic microenvironment, the expression of the Bcl11b transcription factor is induced in thymic progenitors and this determines a full T-cell commitment with activation of T-cell receptor expression

and repression of alternative differentiation cell fates and blocking of stem/progenitor cell properties (Di Santo 2010). The analysis of early T-cell development showed some peculiar findings. The thymus lacks HSCs or other cell types with self-renewing properties, but is continuously seeded by progenitors deriving from bone marrow. The fraction of most immature mouse thymic progenitors displays in vitro and in vivo capacity to generate both T-lymphocytes and myeloid cells (Bell and Bhandoola 2008; Wada et al. 2008). According to these findings it was suggested that the loss of myeloid potential is a relatively late event during T-cell differentiation in the thymus. Luc et al. (2012) have provided evidence at the single cell level that the earliest progenitors in the mouse neonatal thymus possess combined B and T lymphocyte, granulocyte and monocyte, but not erythro-megakaryocytic potential. However, in conflict with these studies, Schlenner et al. (2010) reported that, while the large majority of ETPs originated from IL7RA$^+$ progenitors, only 20 % of thymic neutrophils originated from these progenitors, thus questioning the physiologic relevance of this differentiation pathway.

Studies carried out on human CB progenitors have led to identify CLMPs with a membrane phenotype corresponding to CD34$^+$Thy1$^{low/-}$CD45RA$^+$CD10$^+$CD7$^-$, with a multipotential lymphoid potential (B-, T- and NK lymphocytes), with a myeloid (granulocytes and monocytes) potential and lacking staminal, repopulating activity (Doulatov et al. 2010). The multipotential potential, combined with a myeloid potential of CLMPs, was further confirmed by Goardon et al. (2011) showing that CD34$^+$CD38$^-$Thy1$^-$CD45RA$^+$CD10$^+$CD7$^-$ cells isolated from normal bone marrow have the potential to generate B, T, NK cells, as well as myeloid cells in vivo in NSG mice. Gorgens et al. (2013) identified a cell fraction highly enriched in LMPPs (Lympho-Myeloid Progenitors) with a membrane phenotype CD34$^+$CD133$^+$CD38$^{low/-}$CD45RA$^+$: subdivision of these cells according to CD10 positivity leads to identify a CD10$^+$ fraction lacking granulocyte potential, but retaining lymphoid potential, corresponding to the MLP described by Doulatov, and a CD10$^-$ fraction corresponding to LMPPs. The presence of an early lymphoid-primed human progenitor was obtained by Kohn et al. showing that CD10$^-$CD62L^{high} progenitors isolated from bone marrow have lymphoid and monocytic potential; these cells can be placed at an intermediate stage of differentiation between HSCs and CD34$^+$CD10$^+$ lymphoid progenitors (Kohn et al. 2012).

Some studies have challenged the view that LMMPs possess only lympho-myeloid potential, showing that these cells possess erythro-megakaryocytic potential in vivo (Boyer et al. 2011). However, Boiers et al. (2013) have provided evidence about the existence in mice of lympho-myeloid progenitors emerging during development prior to definitive HSCs and playing a physiologic relevant role in the generation of a lymphoid and myeloid cell progeny. This progenitor (characterized as a cell with an IL7Ralpha$^+$Kit$^+$Flt3$^+$Lin$^-$ membrane phenotype) first appears in yolk sac and contributes physiologically to the establishment of lymphoid and some myeloid components of the immune system. Additional evidence about a physiologic role of early ETPs in promoting granulocytic differentiation at the level of the thymus derives from another recent study (De Obaldia et al. 2013). In fact, these authors have shown that the analysis of various animal models in which ETPs are

absent either for abrogation of the thymic settling or for the inhibition of early thymic development by IL7Ralpha or HES1 genetic knockdown, showed a marked reduction of the thymic granulocytes (De Obaldia et al. 2013).

Signaling via NOTCH receptors is essential for the generation of ETPs in the thymus. NOTCH signaling acts through two different mechanisms, both up-regulating T-cell lineage-specific gene expression and antagonizing alternative differentiation cell fates, as progenitor cell commit to the T-cell lineage. Particularly, the NOTCH-induced transcription factor HES1 acts as a repressor of C/EBPalpha and of the myeloid differentiation program of ETPs (De Obaldia et al. 2014).

Given the complexity of the process of T-cell differentiation from HSCs, it is not surprising that HSC transplantation is followed by a period of immune deficiency due to the paucity of T-cell reconstitution. This problem has stimulated the research on the identification of T lymphocyte progenitors capable of thymus engrafting capacity and of T-cell reconstitution into immunodeficient mice. In this context, both pro-T1 ($CD34^+CD7^+CD5^-$) and pro-T2 ($CD34^+CD7^+CD5^+$) cells were capable of improving and of accelerating HSC-mediated reconstitution of T lymphopoiesis into immunodeficient NOD/SCID mice (Awong et al. 2013).

A recent study has provided evidence about a peculiar mechanism involved in thymic lymphopoiesis and required to maintain a normal, non-neoplastic T-lymphopoiesis. This mechanism is based on the continuous supply of bone marrow-derived progenitors to the thymus: these progenitors replace thymus-resident progenitors. This continuous replacement is based on a progenitor strategy of competition between the bone marrow-derived progenitors and the thymus-resident progenitors for the T-cell growth factor IL-7: under normal conditions and then in the presence of bone marrow-derived progenitors, the availability of IL-7 for thymus-resident progenitors is low and these cells undergo apoptosis. In the absence of incoming bone marrow progenitors, the thymus-resident progenitors proliferate and differentiate, generating T lymphoid cells. However, surprisingly in the absence of a progenitor competition mechanism the endogenous progenitors undergo transformation, generating tumors similar to T-ALLs and with genetic abnormalities, such as NOTCH1 mutations, typical of these leukemias (Martins et al. 2014). Therefore, the physiologic competition between bone marrow-derived progenitors and thymus-resident progenitors, greatly reduces the chances that these last progenitors can undergo malignant transformation.

A key event occurring during B lymphopoiesis is represented by immunoglobulin (Ig) gene rearrangements promoted by the activation of recombination enzymes (RAG-1 and RAG-2 and terminal deoxynucleotidyl transferase promote the D-to-J and V-to-DJ rearrangements at the level Ig heavy chain locus during the differentiation from the CLP to the pre-B stage). At the pre-B-cell differentiation stage, signaling through the pre-B-cell antigen receptor determines the induction of VJ_L rearrangements and allelic exclusion at the Ig heavy chain locus, thus determining the formation of a functional B-cell antigen receptor on the surface of immature B-cells. This rearrangement machinery is timely orchestrated by a number of transcription factors, such as PU.1, PAX5, E2A and EBP, playing a key role in the control of B-lymphopoiesis.

On the other hand, studies on B-lymphopoiesis starting from CLMPs have shown a sequence of events that determines starting from $CD34^{+}CD38^{-}CD45RA^{+}CD10^{-}$ cells the progressive generation of progenitors (pre-pro-B-cells and multilineage CLP/early-B-cells) characterized first by the acquisition of CD19 expression and then CD10 expression; the progenitors thus generated act in turn as precursors for distinct pro-B, pre-B-cells (Sanz et al. 2010). During the early stages of human B lymphopoiesis the level of CD10 expression is an important marker of B-cell commitment and differentiation. In fact, $CD34^{+}CD10^{high}$ cells express CD19 and lymphocyte transcription factors and correspond to loss of myeloid differentiation potential; in contrast, $CD34^{+}CD10^{low}$ cells showed a multiple differentiation potential, being capable of generating lymphocytes, plasmocytoid and conventional dendritic cells and myeloid cells (Ichii et al. 2010).

2 B-cell Acute Lymphoblastic Leukemias; Molecular Abnormalities

The large majority of B-ALLs display chromosomal abnormalities detectable by conventional cytogenetic studies. According to these abnormalities, B-ALLs can be subdivided in: high hyperploidy, consisting in the gain of at least five chromosomes and representing the most frequent pediatric B-ALL subtype; a rare hypodiploid B-ALL subtype with <44 chromosomes; a spectrum of chromosomal translocations, including: t(12;21)(p13;q22) encoding the fusion protein ETV6-RUNX1 (TEL-AML1) (20–25 % of pediatric B-ALLs); t(9;22)(q34;q11.2) encoding BCR-ABL1 (representing about 2 % of pediatric B-ALLs); t(1;19)(q23;p13.3) encoding TCF3-PBX1 (E2A-PBX1) (about 4 % of pediatric B-ALLs); rearrangement of MLL at 11q23 with different partner genes, the most common being AF4 (about 6 % of pediatric B-ALLs); rearrangement of CRLF2 with P2RY8 or with the immunoglobulin heavy chain locus (occurring in B-progenitor cell B-ALLs); rearrangement of IGH locus with a wide range of partner genes, including IL3, CEBPE, BCL2, EPOR, ID4 (Table 14.1).

2.1 ETV6-RUNX1 B-ALLs

Approximately 25 % of B-ALLs are characterized by a balanced t(12;21) chromosomal translocation that generates the ETV6-RUNX1 fusion gene. This B-ALL subtype, associated with a favorable prognosis, was the object of intensive studies showing that the ETV6-RUNX1 translocation is the initiating key event of this B-ALL and occurs prenatally in a committed B-cell progenitor (Greaves and Wiemels 2003). However, the fusion ETV6-RUNX1 gene was not sufficient by itself to induce the full development of overt leukemia and a number of studies have

Table 14.1 Genetic abnormalities found in B-ALLs and their prognosis

Main genetic abnormality	Genes specifically involved	Additional genetic abnormalities and biological features	Prognosis
Hyperdiploidy (>50 and <66 chromosomes). About 30 % of pediatric B-ALL; about 10–13 % of adult B-ALL	Chromosomes X, 4, 6, 10, 14, 17, 18 triploid Chromosome 21 tetraploid	CAN of several genes, including IKZF1, PAX5, ETV6, CDKN2A, RB1, TCF3 Mutations in MAPK signaling pathway (HRAS, NRAS, FLT3, PTPN11) BCR-ABL in adult patients (14 %)	Good
Hypodiploidy (<46 chromosomes) High hypodiploidy (40–45 chromosomes) Low hypodiploidy (33–39 chromosomes) Very low hypodiploidy (30–32 chromosomes) Near Haploidy (23–29 chromosomes)	Chromosomes 3, 7, 17, 15, 16 13, 9, 4, 12 and 20 are the most frequently lost	In low-hypodiploid ALLs: TP53, IKZF2, RB1 Near-haploid ALLs: RTK, Ras pathway, IKZF3	Adverse clinical outcome, low and near haploid karyotype, particularly
t(12;21)(p13;q22) About 25 % of pediatric B-ALL	ETV6-RUNX1 (TEL-AML1)	Low number of somatic mutations (7/leukemia) and none of them was recurrent CNAs: ETV6 (54 %), CDKN2A (22 %), PAX5 (22 %), BTG1 (6 %), TBL1XR1, RAG1, RAG2	Good
t(9;22)(q34;q11) 25–30 % of adult B-ALL; 3–5 % of pediatric B-ALL	BCR/ABL1 (Philadelphia chromosome, $Ph^{+)}$	Frequent (>60 %) deletion of the transcription factor Ikaros (IKZF1). Frequent (48 %) deletion of CDKN2A. CRLF2 genomic rearrangement. Ras signaling pathway mutations. TCF3 and PAX5 (45 %) deletions	Poor in adult Intermediate in children
t(1;19)(q23;p13) balanced and unbalanced translocations. About 3 % of pediatric B-ALL	TCF3-PBX1	1q gain; CAN of CDKN2A/B (23 %) and PAX5 (18 %); alterations of B-cell pathway genes	Intermediate

(continued)

Table 14.1 (continued)

Main genetic abnormality	Genes specifically involved	Additional genetic abnormalities and biological features	Prognosis
BCR-ABL1 like 10–15 % of pediatric B-ALL		Gene expression profile similar to BCR-ABL1-positive B-ALLs. About 50 % of these B-ALLs have CRLF2 rearrangements with JAK1/2 mutations. Mutations of ABL1, ABL2, EPOR, JAK2 and PDGFRB in cases without CRLF2 rearrangements. Frequent BTG1 deletion (31 %)	Poor in adult Intermediate in children
MLL rearrangements (1q23) 6 % of pediatric ALL, particularly frequent among infants t(4;11)(q21;23) t(9;11)(p22q23) t(11;19)(q23;p13.3) t(10;11)(p13-14;q14-21)	MLL-AFF(AF4) MLL-MLLT3(AF9) MLL-ENL MLL-MLLT10(AF10)	Additional genetic mutations are infrequent in these B-ALLs. CNAs are very rare, with the exception of CDKN2A (31 %). KRAS or NRAS mutations in 16 % of patients. FLT3 mutations in a part of patients	Poor
B-ALL with ERG deletion About 3 % of pediatric B-ALLs	ERG	Frequent IKZF1 deletion (38 % of patients), CDKN2A/B (20 %), PAX5 (12 %), ETV6 (3 %), BTG1 (6 %)	Good
B-ALL with CRLF2 rearrangement (Cytokine Receptor Like Factor 2) 7 % of B-ALLs; 53 % of Down patients with ALL	CRLF2, CRLF2-P2RY8, CRLF2-IGH	JAK1 and JAK2 mutations; deletion or mutation of IKZF1	Poor/intermediate
iAMP2 3 % of pediatric B-ALL	Amplification and deletion of parts of chromosome 21	Frequent RB1 (39 %), PAR1 (30 %), ETV6 (21 %) and IKZF1 (25 %) deletions. P2RY8-CRLF2 translocation (38 %)	Poor/intermediate

shown that additional mutations are required for the development of this B-ALL. Genome profiling studies have shown that the additional genetic events occurring in ETV6-RUNX1 B-ALLs are mainly represented by copy number aberrations (CNAs), mainly deletions, affecting genes involved in the control of B-lymphocyte proliferation, development and differentiation, such as CDKN2A, PAX5, BTG1, TBL1XR1, RAG1, RAG2 and the WT copy of ETV6 (Mullighan et al. 2007). These CNAs are related to a mechanism of aberrant RAG endonuclease targeting the promoters, enhancers and first exons of genes that normally regulate B-cell differentiation and represent the largely more frequent secondary events occurring in ETV6-RUNX1 B-ALL, while point mutations are much more rare events (Papamannuil et al. 2014).

2.2 Hyperdiploid B-ALLs

Hyperdiploid B-ALLs are the commonest subtype of B-ALLs in childhood, accounting for about 30 % of all pediatric B-ALLs, include leukemias with >50 and <66 chromosomes and usually have a good prognosis, due to a good response to standard therapy. These leukemias do not display a random pattern of chromosome gain, usually involving gains of chromosomes X, 4, 6, 10, 14, 17, 18 and 21 and all these gains are triploid, with the exception of chromosome 21 gain that is tetraploid. In some cases, the chromosome gains are associated with the classical translocations observed in B-ALLs and these forms are associated with a less good prognosis. Genomic studies have shown some recurrent abnormalities in these B-ALLs, including copy number alterations at the level of some genes, including CDKN2A and mutations in MAPK signaling pathway (KRAS, PTPN11 and FLT3) and in histone-modifying CREB-binding protein gene (particularly in relapsing cases) (Inthal et al. 2012; Paulsson et al. 2008, 2010). Case et al. (2008) showed that 58 % of hyperdiploid B-ALLs exhibited mutations of genes affecting the RAS pathway, the more common being mutations at the level of KRAS or NRAS.

High hyperploid B-ALL is less frequent among adolescent and adult B-ALL patients, its frequency being estimated around 10 %. The presence of two primary genetic aberrations within the same clone is B-ALL is rare, but the contemporaneous presence of hyperploidy and the BCR/ABL translocation is a notable exception. The frequency of these "double-hit" B-ALL in pediatric patients is very low, due to the low frequency of BCR-ABL-positive in children, but is clearly higher in adult B-ALL where the incidence of BCR-ABL-positive B-ALLs is markedly more pronounced (14 % of adult BCR-ABL$^+$ B-ALLs are hyperdiploid and 13 % of adult BCR-ABL$^-$ B-ALLs are hyperdiploid). The comparison of the pattern of chromosome gains in the two groups of hyperdiploid B-ALLs was comparable, with the exception of trisomy of chromosome 2 which was much more frequent among Ph$^+$ hyperdiploid B-ALLs, than Ph$^-$ hyperdiploid B-ALLs (Chilton et al. 2014).

Subclonal analysis provided evidence that the numerical chromosome aberrations are the primary events and arose before structural events, suggesting a stepwise evolution of the leukemic clone (Paulsson et al. 2010).

2.3 Hypodiploid B-ALLs

Hypodiploidy is observed in 5–8 % of ALLs and can be subdivided into: (a) high hypodiploidy (40–45 chromosomes), (b) low hypodiploidy (33–39 chromosomes); very low hypodiploidy (30–32 chromosomes) and near haploidy (23–29 chromosomes), associated with distinct genetic and clinical features. The majority of hypodiploid patients has 45 chromosomes; low hypodiploidy and near haploidy B-ALLs are rare and are associated with a very negative prognosis.

A recent study provided fundamental information about the genomic landscape of hypodiploid B-ALLs, showing that these ALLs form a peculiar subtype of B-ALLs, distinct from other B-ALL subtypes. In fact, this study showed that low hypodiploid B-ALLs have a very frequent mutation of TP53 and frequent inactivating mutations (53 %) of the IKAROS family gene IKZF2 (HELIOS) and of the retinoblastoma gene (RB1, 41 %); near-haploid ALLs with 24–31 chromosomes harbor genetic abnormalities at the level of the Ras signaling pathway (71 %) and of the IKAROS family gene IKZF3 (AIOLOS). Very interestingly, the TP53 mutation found in low-hypodiploid B-ALL cells was also observed in matched non-tumor cells, suggesting germline inheritance; according to this observation it was suggested that low hypodiploid B-ALL could represent a manifestation of Li-Fraumeni syndrome (Holmfeldt et al. 2013). It is of interest to note that in the majority of these patients with TP53 mutated, both TP53 alleles are mutated or one is mutated and the other one is deleted (Stengel et al. 2014). Other studies have confirmed the very frequent (93 %) occurrence of TP53 mutation in low hyperdiploid B-ALLs; importantly, in these B-ALLs the normal TP53 allele was lost due to monosomy 17 (Muhlbacher et al. 2014).

2.4 BCR-ABL1 ALLs

This subgroup of B-ALLs is characterized by the formation of the BCR-ABL1 fusion transcript due to der(22) of the t(9;22)(q34;q11) translocation, or Philadelphia (Ph) chromosome. BCR-ABL1 represents about 25–30 % of adult B-ALLs and 3–5 % of pediatric B-ALLs. About 30 % of B-ALL patients display the p210 BCR-ABL fusion protein (formed by the breakpoint in the middle of BCR), while the remaining 70 % display the p190 BCR-ABL fusion protein (resulting from breakpoints in the BCR minor cluster region within the BCR intron 1). Both fusion proteins have a transforming potential of hematopoietic cells and induce a syndrome similar to CML in mice. Genome-wide analysis of B-ALLs showed that 83 % of these

leukemias display deletion of transcription factor Ikaros (IKZF1), a master regulator of B-cell differentiation. The IKZF1 deletions resulted in haploinsufficiency, expression of a dominant negative form of Ikaros or the complete loss of Ikaros expression (Mullighan et al. 2008b). The presence of Ikaros deletions represent an important prognostic factor of BCR-ABL B-ALLs both in pediatric and adult patients because they are associated with a poorer outcome and resistance to treatment with the BCR-ABL TKIImatinib.

2.5 BCR-ABL1-Like ALLs

IKZF1 alterations are observed also in a group of B-ALL patients, not displaying BCR-ABL1 translocation; these B-ALLs correspond to about 15 % of all pediatric B-ALLs and 30 % of adult B-ALLs, have a poor outcome and usually exhibit a gene expression profile similar to BCR-ABL1-positive ALLs and these cases are referred as Ph-like ALLs. Approximately 50 % of Ph-like ALL patients have rearrangements of CRLF2, with concomitant JAK 1 or 2 mutations. Transcriptome and wide-genome sequencing studies have shown that Ph-like B-ALLs without CRLF2 rearrangements frequently display genetic abnormalities activating cytokine receptors and tyrosine kinases, such as ABL1, ABL2, EPOR, JAK2 and PDGFRB. The deregulation of these kinases derives from fusion events involving these genes and resulting in a deregulated tyrosine kinase activity. About 20 % of Ph-like B-ALLs lack a chimeric fusion, but possess activating mutations of either IL7R, FLT3 or focal deletions of SH2B3 encoding LNK (Roberts et al. 2012).

Recently, Roberts et al. (2014a) have published the results of the genomic profiling of 154 patients with Ph-like B-ALLs. This large genomic screening allowed to establish that 91 % of patients with Ph-like ALLs display kinase-activating lesions consisting either in rearrangements involving ABL1, ABL2, CRLF2, CSF1R, EPOR, JAK2, NTRK3, PDGFRB, PTK2B, TSLP or TRYK2 or sequence mutations involving FLT3, IL7R or SH2B3. Alterations of ABL1, ABL2, CSF1R, JAK2 and PDGFRB resulted in Stat5 activation and cytokine-independent proliferation. Importantly, some of these abnormalities are clearly sensitive to available kinase inhibitors. Given these observations, it seemed logical to perform a clinical approach of individually treating Ph-like ALLs according to their mutational status at the level of cytokine receptors or tyrosine kinase. This approach considerably improved the outcome of this type of B-ALL patients (Roberts et al. 2014b).

2.6 B-ALLs with ERG Deletion

Recently, a new subtype of B-ALL, characterized by deletion of the ETS-family transcription factor ERG, was identified. In this B-ALL subtype, associated with a peculiar gene expression profile, ERG deletions involve an internal set of exons,

resulting in loss of the central inhibitory domain and expression of truncated ERG isoform that acts as competitive inhibitor of WT-ERG (Harvey et al. 2010). A more recent study based on the analysis of a large cohort of patients showed that the frequency of B-ALLs with ERG deletions correspond to 3.2 %. ERG deletion was mutually exclusive with other genetic lesions and was characterized by aberrant CD2 expression and frequent IKZF1 deletions (Clappier et al. 2014). In spite of the presence in this B-ALL subtype of frequent IKZF1 deletions, the prognosis was good.

2.7 MLL-Rearranged B-ALLs

MLL-rearranged B-ALLs represent about 6 % of pediatric ALLs, are characterized by a poor prognosis and are particularly frequent in infants, where they occur in about two third of infants with ALLs. Many partner genes of MLL rearrangements have been identified, but the more frequent is AF4, observed in about 50 % of cases. MLL-rearranged B-ALLs are characterized by a peculiar pattern of gene expression characterized by high expression of class I HOX genes, cooperating together with MLL fusions in inducing leukemia and in maintaining a stem cell-like state of differentiation (Faber et al. 2009). It is important to point out that MLL translocations arise in utero and rapidly lead to the development of overt leukemia, at birth or shortly after.

At variance with other B-ALL subtypes, additional genetic mutations are infrequent in MLL-rearranged B-ALLs. Particularly, copy number alterations are very rare in MLL-rearranged B-ALLs at diagnosis (Bardini et al. 2010). Ras mutations are observed in a minority of B-ALL patients with MLL rearrangements (about 16 % of these patients display either NRAS or KRAS mutated) (Driessen et al. 2013). Furthermore, FLT3 kinase domain mutations are also reported in a variable fraction of infants with MLL-AF4 ALLs. This conclusion was directly supported by whole genome sequencing of MLL-AF4 pro-B ALLs, showing the absence of CNAs in these ALLs and the occurrence of very few somatic mutations (a mean of 5 mutations) (Dobbins et al. 2013).

MLL-rearranged B-ALLs have a peculiar epigenetic profile, with signatures of cytosine, microRNA and H3K79 methylation differing from either types of B-ALLs. The increased H3K79 methylation derives from the enhanced activity of the histone methyltransferase DOT1L. Importantly, suppression of DOT1L expression into human and murine MLL-AF4 leukemic cells determines an inhibition of the MLL-induced expression program, differentiation and/or apoptosis of leukemic cells and blockade of leukemogenesis (Krivstov et al. 2008; Jo et al. 2011).

Recent studies showed that MLL fusion proteins are regulated in leukemia cells via proteolysis by the proteasome; furthermore, at variance with other oncoproteins, MLL-fusion proteins are expressed in leukemic cells at low levels. The addition of Bortezomib, a proteasome inhibitor, induced a clear increase of MLL-AF4 protein levels and apoptosis of leukemic cells through activation of the extrinsic apoptotic

pathway. Specific gene silencing experiments provided evidence that the high sensitivity of this ALL subtype is specifically dependent on the presence of the MLL-AF4 fusion protein (Liu et al. 2014b).

2.8 B-ALL with CRLF2 Rearrangement

Some B-ALLs were characterized by rearrangements involving the Cytokine Receptor-Like Factor 2 (CRLF2), also known as Thymic Stromal-Derived Lymphopoietin (TSLP) Receptor. Together with the IL-7Ralpha chain, CRLF2 forms a heterodimeric receptor for TSLP. A first type of CRLF2 abnormality was identified by Mullighan and coworkers in 2008, showing a recurrent interstitial deletion of pseudoautosomal region 1 of chromosome X and Y in B-ALL that juxtaposes the coding region of CRLF2 with noncoding exon of P2RY8 (purinergic receptor gene): the CRLF2-P2RY8 fusion was observed in 7 % of patients with B-ALL and in 53 % of Down patients with ALL (Mullighan et al. 2008a). Subsequent gene expression profiling studies have shown that 14 % of high-risk B-ALLs display hyperexpression of CRLF2; all these cases harbored a rearrangement of the CRLF2 gene: 32 % had the CRLF2-P2RY8 fusion and 62 % had a translocation of the immunoglobulin heavy chain gene IgH on 14q32 to CRLF2 (Harvey et al. 2010). CRLF2 rearrangements were associated with activating mutations of JAK1 or JAK2, deletion or mutation of IKZF1, and a peculiar Hispanic/Latin ethnicity (Harvey et al. 2010).

Less frequently, CRLF2 harbors a Phe232Cys gain-of-function mutation that promotes constitutive dimerization and cytokine-independent growth (Shochat et al. 2014). It is important to underline that CRLF2 rearrangements are frequently (up to 50 %) observed in BCR-ABL1-like ALLs (Harvey et al. 2010). B-ALL with rearranged CRLF2 displays a transcriptional signature that greatly overlaps with a BCR/ABL signature and is enriched for genes involved in cytokine receptor and JAK-STAT signaling; furthermore, these ALLs are associated with a poor outcome (Yoda et al. 2010). As above mentioned, about 50 % of CRLF2-rearranged B-ALLs display activating mutations of JAK1 and JAK2; in non-Down syndrome ALLs, CDLF2 alterations and JAK2 mutations are associated with IKZF1 deletion/mutation.

Recently, it was reported the full-exome sequencing of Down syndrome B-ALLs, showing the frequent occurrence of some driver mutations including RAS mutations (36 % of cases), JAK2 mutations (29 % of cases) or CRLF2-P2RY8 fusions (34 %); RAS mutations were shown to be mutually exclusive with JAK2 mutations. Clonal architecture analysis suggested that CRLF2 rearrangement represents the initial oncogenic event, followed by JAK2 or RAS mutations as secondary events driving subclonal expansions (Nikolaev et al. 2014).

2.9 B-ALLs with MYC Translocations

Chromosomal rearrangements involving the MYC gene, located on band 8q24, are a typical characteristic cytogenetic abnormality of Burkitt lymphoma and several subsets of other mature B-cell neoplasms. The MYC rearrangement determines a major dysregulation of the MYC oncogene and plays a key role in genesis of these diseases by juxtaposing the MYC gene to immunoglobulin genes. The major cytogenetic abnormality observed in Burkitt lymphoma is the MYC-immunoglobulin heavy chain gene (IGH) rearrangement t(8;14)(q34;q32), followed by MYC-rearrangement t(8;22)(q24;q11). Although MYC rearrangements are mainly found in mature B-cell lymphoid neoplasias, cases of B-ALL carrying the MYC rearrangement are observed. Around 3 % of adult B-ALLs show the chromosomal translocation t(8;14)(q34;q32) and display a mature B-ALL or Burkitt-type ALL immunophenotype. In these B-ALLs, the location of the chromosomal breaks in the IGH locus occur at the level of the joining and the eight different switch regions of this gene (Burmeister et al. 2013). Immunophenotypic features of these B-ALLs are compatible with Burkitt type ALL/Burkitt lymphoma (i.e., $CD34^+$, $CD19^+$, $CD22^+$, $HLA\text{-}DR^+$, $CD10^+$, sIg^+, TdT^-). The majority (>60 %) of adult B-ALLs with MYC rearranged display TP53 mutations and have a poor outcome (Stengel et al. 2014). Rare cases display the combined translocations of both MYC and MLL translocations (Meeker et al. 2011). The majority of B-ALL with Burkitt-type MYC rearrangements have a mature B-cell phenotype; however, some cases display a FAB L3 morphology and a B-precursor immunophenotype and lack to express surface Igs (Navid et al. 1999).

In some patients a Burkitt-type ALL was observed in association with the translocation t(14;18)(q32;q21), typical of follicular lymphomas: in these rare B-ALL patients this translocation was found in association with various types of MYC translocations, such as the classical Burkitt t(8;14)(q34;q32) or der(14)t(14;19) or the Burkitt variant t(8;22)(q34;q11) or the non-Burkitt MYC rearrangement t(8;9)(q24;p13); these B-ALLs are associated with a very negative prognosis (Dunphy et al. 2003; D'Achille et al. 2006).

2.10 iAMP21 B-ALL

About 2 % of B-ALLs show an intrachromosomal amplification of one copy of chromosome 21, iAMAP2, which defines a distinct B-ALL subgroup with prognostic and therapeutic implications. Initial studies have shown the complex nature of chromosome 21 structure in these patients with a common 6.6 mb-common region of amplification on chromosome 21 containing RUNX1 and c common region of deletion at the telomere. Gene profiling studies have failed to detect in these patients consistent abnormalities of relevant genes present on chromosome 21. More recently, studies have greatly contributed to understand the molecular pathogenesis

of these B-ALLs showing numerous copy number alterations mostly targeting chromosome 21 and involving deletion of IKZF1 (22 %), CDKN2A/B (17 %), PAX 5 (8 %), ETV6 (19 %) and RB1 (37 %) (Rand et al. 2011). Furthermore, the P2RY8-CRLF2 fusion was observed in 38 % of iAMP21 patients (Russell et al. 2009). Analysis of the clonal architecture of these B-ALLs showed that the various abnormalities are secondary to chromosome 21 rearrangements (Rand et al. 2011). Initial clinical studies, where the iAMP2 B-ALLs were treated with standard protocols showed a poor outcome; the outcome of these leukemias, however, clearly improved when intensified treatments were introduced for high-risk B-ALLs (Harrison et al. 2014).

Interestingly, a recent study showed that 3 % of iAMP21 B-ALLs display a constitutional Robertsonian translocation between chromosomes 15 and 21, rob (15;21) (q10;q10). Individuals born with this rare constitutional translocation have about 2,700 fold increased risk of developing iAMP21 B-ALL compared to the general population. In these cases, amplification is initiated by a chromothripsis event involving both sister chromatids of the Robertsonian chromosome; subsequently, duplication of the entire chromosome 21 occurs. In sporadic iAMP21 cases, breakage-fusion-bridge cycles are typically the initiating event, frequently followed by chromothripsis (Li et al. 2014).

2.11 Clinical-Molecular Classification of B-ALLs

Current risk classification of B-ALLs included several pretreatment clinical features including white blood cell count, age and the presence or the absence of recurrent cytogenetic abnormalities and analysis of minimal residual disease at the end of induction therapy and classifies these leukemias into four different groups: low, standard/intermediate, high and very high (Schultz et al. 2007). Very high-risk B-ALLs corresponded to about 4–5 % of pediatric B-ALLs and included leukemias with BCR-ABL translocation or hypodiploidy, failure to achieve a complete remission at the end of induction therapy (with >25 % of leukemic blasts). Low-risk B-ALLs represented about 27–30 % of all B-ALLs and include leukemias with the t(12;21)(TEL/AML1) or simultaneous trisomies of chromosomes 4, 10 and 17 (hyperdiploid B-ALLs).

Recently, a new simplified risk stratification of pediatric B-ALLs was proposed, based on the integration of cytogenetic and genomic data (the genomic data were related to the major CNAs observed in B-ALLs, concerning eight genes, IKZF1, CDKN2A/B, PAR1, BTG1, EBF1, PAX5, ETV6 and RB1). This classification identified two groups: a god-risk group included patients with ETV6-RUNX1, high hyperploidy, normal copy-number status for all eight genes, isolated deletions affecting ETV6/PAX5/BTG1, and ETV6 deletions with a single additional deletion of BTG1/PAX5/CDKN2A/B; a poor risk group including all the other genetic features. The clinical data observed on >1,500 B-ALL patients supported a significant

difference between the two groups of patients at the level of event-free survival (94 % vs 79 %) and relapse rate (4 % vs 17 %) (Moorman et al. 2014).

Harvey et al. (2010) have performed a gene expression profiling study to attempt a better characterization and classification of high-risk pediatric B-ALLs. Unsupervised clustering of gene expression profiling showed 8 unique cluster groups with these high-risk B-ALLs. Only clusters 1 and 2, corresponding each to about 10–11 % of total high-risk B-ALLs, were associated with known chromosomal translocations: cluster 1 with MLL rearrangements and cluster 2 with t(1;19) (TCF3-PBX1). Clusters 3 and 4, corresponding each at about 5–6 % of total B-ALLs, are characterized by the presence of a very high frequency of CDKN2A deletions (80–90 % of cases) and by the frequent (cluster 3, 25 %) or very frequent (cluster 4, 85 %) PAX5 deletions; cluster 3 displays a relapse-free survival (RFS) at 4 years in the average, while cluster 4 displays a RFS lower than then average of the whole high-risk group. Cluster 5 is a small group corresponding at about 5 % of these patients and shows frequent ETV6 (40 %) and IKZF1 (30 %) deletions. Cluster 6 corresponds to 10 % of high-risk B-ALLs and is characterized by frequent (40 %) ERG deletions and is the group displaying the best prognosis, with a RFS at 4 years of 94 % of patients. The cluster 7 represents the largest group (about 35 %) of high-risk B-ALLs and is characterized by the presence of multiple CNAa, involving CDKN2A, IKZF1, PAX5 and also ETV6 and IL-3RA; about 10 % of these B-ALLs display CRLF2 rearrangements; these patients display a RFS moderately lower than the average of the whole high-risk B-ALL group (Harvey et al. 2010). The group 8 corresponds at about 11 % of high-risk B-ALLs and is characterized by the very frequent (75 %) CRLF2 rearrangements and by the presence of multiple CNAs, particularly frequent IKZF1 (>90 %) and CDKN2A/B (about 60 %) deletions, and by frequent (50 %) JAK1/JAK2 mutations; this group is associated at a poor prognosis with a RFS markedly lower (23 % at 4 years) than the average (66 % at 4 years) of the whole high-risk B-ALL group.

2.12 Relapsed B-ALLs

Despite intensive chemotherapy, about 20 % of pediatric patients and >50 % of adult patients with B-ALL do not achieve a complete remission or relapse after chemotherapy. As above mentioned, several chromosomal alterations, such as BCR-ABL1 and MLL rearrangements are associated with high rates of relapse; however, all B-ALL subtypes may relapse, including B-ALL subtypes with favorable prognosis. Since the prognosis of relapsed B-ALL patients is usually poor, there is a consistent interest to characterize at molecular and clonal/subclonal level relapsed B-ALL. Thus, several studies have performed microarray profiling studies comparing matched leukemic samples at diagnosis and relapse to identify new mutations occurring only at relapse and to determine the genetic heterogeneity at clonal level of B-ALLs and to understand how this heterogeneity may affect B-ALL relapse. The initial studies involving the matched analysis of B-ALL patients

showed that the majority of B-ALLs display significant changes in the spectrum of genetic alterations from the diagnosis to the relapse and that many of these alterations relapse-acquired, such as those occurring at the level of IKZF1 and CDKN2A/B, are in fact present at low level at diagnosis, confined to rare tumor subclone (Yang et al. 2008). Mullighan et al. (2011) have sequenced 300 relevant genes in matched diagnosis and relapse B-ALL samples. Using this approach they identified 52 somatic mutations in 32 genes, many of which seem to be acquired at relapse, and particularly at the level of the transcriptional co-activators CREBBP and NCOR1, come transcription factors (ERG, SPI1, TCF4 and TCF7L2) and many components of the Ras signaling pathway. Particularly, they showed that 18 % of released B-ALL displayed sequence or deletion mutations of CREBBP and that these alterations were present only in a part of these patients at diagnosis. Inthal et al. (2012) observed a very high incidence (63 % of cases) of CREBBP mutations among relapsing high hyperploid B-ALL, while these mutations were observed on only 19 % of these relapsing patients at diagnosis. Interestingly, CREBBP mutations at diagnosis were not observed in long-term survivor patients with high hyperploid B-ALLs.

A recent study by Meyer and coworkers provided evidence that about 10 % of relapsed B-ALL pediatric patients display relapse-specific mutations at the level of the 5′-nuceotidase NT5C2, an enzyme that is responsible for the inactivation of nucleoside-analog chemotherapy drugs. These mutations conferred increased enzymatic activity and resistance to treatment with nucleoside analog therapies (Meyer et al. 2013). Irving and coworkers observed a high prevalence (37 % of cases) of somatic mutations activating the Ras pathway (KRAS, NRAS, FLT3 and PTPN11) at the level of a large population of relapsing pediatric B-ALL patients (Irving et al. 2014). Using sensitive allelic specific assays it was possible to demonstrate the existence of low-level mutated subpopulations in the majority of these patients at diagnosis (Irving et al. 2014). Mar et al. (2014) have analyzed a group of relapsing pediatric B-ALL patients and through analysis with matched diagnosis samples they showed that the somatic mutations in epigenetic regulators, such as CREBBP, KDM6A, MLL2, SETD2 and MSH6 are enriched at relapse. They interpreted these findings suggesting that therapy may have applied a selective pressure to acquire or select for rare subclones possessing these mutations.

3 T-cell Acute Lymphoblastic Leukemias; Molecular Abnormalities

T-cell acute lymphoblastic leukemias (T-ALLs) are leukemic processes involving the uncontrolled proliferation of T-cell progenitors/precursors. T-ALLs account for about 25 % of adult ALLs and 10–15 % of pediatric leukemias. At the clinical level, patients with T-ALLs show diffuse BM infiltration by immature T lymphoblasts, mediastinal masses associated to pleural effusions, high white blood cell counts. The prognosis of T-ALLs has improved in the last years due to the development of specific chemotherapy-based treatments; however, the current curative treatment

rate does bypass 75 % in children and 50 % in adults. Gene expression profiling studies have shown the existence of three main types of T-ALL subtypes, indicating three different stages of differentiation at which T-ALL blasts are blocked: (a) early immature T-ALLs indicating an early block in T-cell differentiation at a very early stage; (b) early cortical T-ALLs, characterized by positivity for CD1a, CD4 and CD8 and usually associated with activation of homeobox genes, such as TLX1 and TLX3, NKX2.1 and NKX2.2; (c) late cortical thymocytes expressing CD4, CD8 and CD3 and usually showing activation of the TAL1 transcription factor (Ferrando et al. 2002).

Early immature T-LL is characterized by the specific immunophenotype $CD1a^{-}$, $CD8^{-}$ and $CD5^{weak/-}$, with stem cell or myeloid marker expression. The early immature T-ALL subgroup represents a peculiar subtype of T-ALL, with a unique genetic basis, as supported by various lines of evidence: (i) expression of LYL1 oncogene and co-expression of LMO2; (ii) high prevalence of 5q,13q and 11q chromosomal deletions and absence of deletions of the short arm of chromosome 9; (iii) expression of the stem/progenitor marker CD34 and of the myeloid markers CD33 and CD13 (Ferrando et al. 2002). These observations were confirmed in more recent studies: Homminga et al. (2011) in a large microarray analysis of T-ALLs identified an immature cluster, largely corresponding to the early immature T-ALL subgroup; Coustan-Smith et al. (2009) defined a subgroup of T-ALLs, characterized by the absent expression of CD4, CD13, CD33, CD11b and named these leukemias subgroup as ETP T-ALLs. Recent studies have provided a molecular characterization of the fine genetic defects occurring in early immature T-ALLs. First, Homminga et al. (2011) have reported in these T-ALLs the recurrent rearrangements, resulting in overexpression of the MEGFC2 gene, encoding a key transcriptional regulator of lymphoid development, highly expressed only in immature thymocytes. Second, Zhang et al. (2012) have carried out a fundamental study reporting whole-sequence analysis of 52 ETP T-ALLs and have described the main genetic alterations occurring in this T-ALL subgroup: (i) high frequency (67 %) of activating mutations of cytokine receptor pathways and RAS signaling pathways, including IL7R, FLT3, JAK1, JAK3, SH2B3, NRAS, KRAS and BRAF; (ii) inactivating mutations (58 %) at the level of transcription factors, acting as regulators of hematopoietic differentiation, such as ETV6, RUNX1, IKAROS (IKZF1), GATA3 and EP300; (iii) inactivating mutations (48 %) at the level of genes encoding histone modifiers, such as SETD2, SUZ12, E2H2, EED. Furthermore, some of these T-ALLs displayed multiple genome rearrangements, suggesting the occurrence of genomic instability, in association with alterations of genes related to DNA mismatch repair. It is of interest to note that the mutational spectrum of ETP T-ALLs is similar to myeloid tumors and the global transcriptional profile is that of normal HSCs and myeloid LSCs.

In line with these results, other studies showed that about 50 % of adult immature T-ALLs display mutations of myeloid-specific oncogenes and tumor suppressor genes, including IDH1, IDH2, FLT3, NRAS and DNMT3A; in this study it was also noted that mutations of the ETV6 tumor suppressor gene are particularly frequent (25 %), resulting in the expression of a ETV6 truncated form with dominant negative activity (Van Vlierberghe et al. 2011). A recent study reported a whole exome

sequencing of adult ETP T-ALLs providing several novel and interesting findings: DNMT3A is mutated in 16 % of cases, while this gene was found not mutated in pediatric ETP T-ALLs; FLT3 was found to be mutated in 35 % of these patients; mutations of other epigenetic regulator genes, such as MLL2 (10 %), EZH2 (6 %), SH2B3 (6 %) and SUZ12 (1 %) were also frequent; novel recurrent mutations in the genes FAT1 (25 %), FAT3 (20 %) and DNM2 (35 %) have been identified; finally, PRC2 mutations, frequent in pediatric ETP T-ALLs are rare in adult ETP T-ALLs (Neumann et al. 2013).

Recent studies characterized pediatric T-ALLs in whom induction therapy failed to induce disease remission. A subgroup of these chemotherapy-resistant patients was characterized at molecular level by the absence of biallelic TCRγ locus deletion (ABD), a characteristic of early thymocyte precursors before V(D)J recombination, and aT-cellular level by a T early precursor cell phenotype (Gutierrez et al. 2010). Zuurbier et al. (2014) have recently characterized these ABD T-ALLs at molecular level showing frequent NOTCH1/FBW7 mutations (57 %), but absent WT1, PHF6 and PTEN/AKT mutations.

Studies carried out in the last years have shown that T-ALL development results from a multistep oncogenic process involving the acquisition of multiple somatic genetic abnormalities at the level of the NOTCH signaling pathway, transcription factors, signaling oncogenes and tumor suppressors (reviewed in Van Vlierberghe and Ferrando 2012). The most frequent genetic alterations occurring in T-ALL is represented by the deletion of the CDKN2A locus, present on chromosome 9p22 and occurring in about 70 % of cases: this locus englobes two different tumor suppressor genes, p14/INK4A and p16/ARF, both involved in the control of cell cycle. The activation of the NOTCH signaling pathway is frequently observed in T-ALLs: in fact, 60 % of T-ALLs, display activating mutations of the NOTCH1 (either at the level of the HD domain of this receptor, or of the PEST domain), while 20 % of T-ALL cases exhibit activation of NOTCH1 mediated by mutations of the FBXW7 gene (encoding a protein involved in the control of stability of NOTCH1 and other relevant oncoproteins such as MYC, MCL1, CyclinE) (reviewed in Tosello and Ferrando 2013). Both the physiologic and oncogenic effects of NOTCH1 require translocation of the intracellular portion of the NOTCH1 receptor to the nucleus, where it activates a specific program of gene expression. In this context, NOTCH1 is a key regulator of the proliferation of T-ALL blasts by controlling various genes involved in the control of cell growth. Among these genes a key role is played by c-myc, whose expression is transcriptionally controlled through the binding to an enhancer present at the level of the proximal c-myc promoter (Herranz et al. 2014).

In about 40 % of T-ALLs, chromosomal translocations juxtaposing transcription factors playing a key role in the control of T-cell differentiation and regulatory elements located in proximity of the T-cell receptor genes are observed (reviewed in Van Viberghe and Ferrando 2012). These T-ALL-specific transcription factors acting as oncogenes for the development of this leukemia are: some members of the HLH family of transcription factors, such as LYL1, TAL1; some members of the LIM-only domain (LMO) family, such as LMO1 and LMO2; some members of the homeobox gene family, including some HOXA genes, TLX1/HOXD11 and TLX3/

HOX11L2; some key oncogenes, such as MYB and MYC; TAN1, a truncated, constitutively active form of the NOTCH1 membrane receptor. In other cases, these transcription factors are activated by genetic abnormalities, different from those involving TCR-associated chromosomal abnormalities, such as duplications of the MYB oncogene, small activating deletions of LMO2 and TAL1 and the translocation determining the activation of TLX3/HOX11L2 gene through its juxtaposition near to the BL11B gene locus (reviewed in Van Vlierberghe and Ferrando 2012).

Homminga et al. (2011) have performed an integrated transcriptomic and genomic analysis of T-ALLs, identifying two T-ALL potential subgroups lacking known oncogenic rearrangements and representing about 20 % of pediatric T-ALLs. One of these two subtypes is associated with cortical thymocyte differentiation block and by very frequent overexpression of NKX2-1/NKX2-2, for which genes frequent rearrangements have been observed (in about 60 % of cases). The second subtype was associated with immature cell development, high expression of MEFC2 transcription factor and rearrangements of MEFC2 or of transcription factors directly targeting MEF2C (in about 50 % of cases). Ectopic expression of NKX2-1 of MEFC2 induces oncogenic effects and interferes with T-cell differentiation. A subsequent study clearly showed that MEFC-dysregulated T-ALLs represent a subgroup of ETP T-ALLs and are characterized by an early T-ALL gene signature and have non-rearranged T-cell receptors, in line with their early T-cell differentiation block (Zuurbier et al. 2014).

Additional mutations, occurring at the level of various transcription factor tumor suppressor genes, such as BCL11B, ETV6, LEF1, PHF6, RUNX1 and WT1, have been reported in T-ALLs. Furthermore, genetic alterations at the level of various signaling pathways have been described in T-ALLs, such as: activating mutations of the cytokine receptors IL7R and FLT3; activating mutations of the transducing proteins JAK1 and JAK3; deletions of the PTPN2 gene; activating mutations of the RAS signaling pathway; deletions and mutations of the PTEN gene (reviewed in Van Vlierberghe and Ferrando 2012).

Recent studies have reported mutations of some genes involved in the epigenetic control of gene expression, such as EZH2, EED, SETD2 and SUZ12, thus highlighting a possible role of altered epigenetic regulation in T-cell oncogenesis (Ntziachristos et al. 2012). Finally, a recent study identified three new oncogenic driver genes in T-ALLs. Thus, CNOT3 was identified as a tumor repressor gene mutated in about 8 % of adult T-ALLs; mutations affecting the ribosomal proteins RPL5 and RPL10 have been detected in about 10 % of pediatric T-ALLs (De Keersmaecker et al. 2013). The mechanism through which mutations of ribosomal RPL proteins affect leukemia development is largely unknown; however, a recent study, based on the expression of the mutant Rpl10-R981 in yeast, suggested that T-cellular adaptation to the presence of this mutant implies changes in gene expression that in long-term undermine cellular homeostasis (Sulima et al. 2014).

All these observations have led to propose a molecular classification of T-ALLs which identifies T-ALL subtypes, each characterized by a type of mutation specific and considered a driver event. Thus, according to this molecular classification the mutations occurring in T-ALLs are classified as type A mutations (Driving

oncogenes that characterize and define different genetic T-ALL subgroups) and type B mutations (common genetic abnormalities that can be found in all T-ALL genetic subgroups). According to this classification, six T-ALL genetic subgroups have been identified, TAL/LMO, TLX1, TLX3, HOXA, MYB, ETP (or LYL1), whose main features are reported in Table 14.2.

The prognostic significance of the various type B gene mutations and abnormalities occurring in T-ALLs was recently evaluated in adult T-ALL patients. Homozygous deletion of CDKN2A, mostly present in cortical/mature T-ALLs, was associated with favorable outcome, compared to the rest of T-ALLs not displaying this abnormality. TP53 heterozygous deletion, observed in about 10 % of T-ALL patients, was associated with worse clinical outcome. NOTCH1 and FBW7 mutants, very frequent among cortical/mature T-ALLs, were associated to a better outcome than non-mutant T-ALLs. Similarly, favorable outcome was observed in adult T-ALL patients with heterozygous inactivating mutations or deletions in the BCL11B tumor suppressor gene. In contrast, somatic mutations in genes targeting the epigenetic regulators DNMT3A and IDH1/2, uniquely present in the early immature adult T-ALL group, are associated with negative prognosis (Van Vlierberghe et al. 2013).

Trinquand et al. (2013) have proposed a simplified classification of adult T-ALL, based on the presence or not in these leukemias of NOTCH1/FBXW7 mutations: the absence of these mutations was found to be associated with a poor prognosis. The group of NOTCH1/FBXW7 mutations was associated with a good prognosis at the condition that in these leukemias are absent KRAS, NRAS and PTEN mutations. According to this classification, about 50 % of adult T-ALLs are predicted to have a "good" outcome and about 50 % a poor outcome. Grossmann et al. (2013) have recently shown the negative impact of RUNX1 and DNMT3A mutations at the level of the early T-ALLs: the presence of these mutations was associated with a short survival.

3.1 Relapsed T-ALLs

The problem of relapsed T-ALLs is a major problem because these ALLs are usually more resistant to treatment than B-ALLs and exhibit a greater tendency to relapse. Relapsed T-ALLs have a poor prognosis. The large majority of T-ALL patients either relapse on-therapy or immediately after or within 2 years after the end of treatment; only about 10 % of these patients relapse 2.5 years after the end of treatment. In an initial study, Szczepanski et al. (2003) have used clonal T-cell receptor gene rearrangements to study the clonal derivation of relapsed T-ALLs. In the majority of cases clonal TCR rearrangements in paired T-ALL specimens were similar at diagnosis and at relapse, thus suggesting that the relapsing clone evolved from the same leukemic clone present at diagnosis. However, in two late-relapsing patients it was noted that the TCR rearrangements observed at diagnosis and at relapse are completely different, thus suggesting that the relapsing clone evolved

Table 14.2 T-ALL genetic subgroups with the main features

Subgroup (frequency, %)	Rearrangement	Gene involved	Additional genetic abnormalities	T-cell differentiation block
TAL/LMO (30–35 %)	t(1;14)(p32;q11)/1p32 deletion t(1;7)(p32;q34)/1p32 deletion t(7;9)(q34;p32) t(11;14)(p15;q11) t(7;11)(q34;p15) t(11;14)(p13;q11)/11p13 deletion t(7;11)(p34;p13)/11p13 deletion	TAL1 (SIL/TAL1) TAL1 (SIL/TAL1) TAL2 LMO1 LMO1 LMO2 LMO2	NOTCH pathway mutation (50 % of cases) Frequent PTEN mutation/deletion (30–40 % of cases) Frequent MYC translocations (17 % of cases) Absent IL-7R mutations CDKN2A deletion (53 %) CASP8 deletion	Pre-αβ/TCR-αβ (Early T/Cortical) Immature/Pre-αβ/TCR-αβ
TLX1 (8 %)	t(10;14)(q24;q11) t(7;10)(q34;q24)	TLX1/HOX11	PTPN2 deletion (about 50 % of cases) Absent IL-7R. Frequent PHF6 mutation (43 %) Frequent BCL11B mutation (50 %) CDKN2A deletion (89 %)	Immature/Pre-αβ The capacity to block T-cell differentiation is due to interaction with Ets1 and block of TCR gene expression
TLX3 (24 %)	t(5;14)(q35;q32)	TLX3/HOX11L2	Frequent WT1 gene mutations (35 %) Absent PTEN mutation/deletion Frequent IL-7R mutations (16 %) Frequent PHF6 mutations (43 %) Frequent BCL11B mutation (25 %) CDKN2A deletion (78 %) RB1 deletion	Immature/Pre-αβ The capacity to block T-cell differentiation is due to interaction with Ets1 and block of TCR gene expression

HOXA (15 %)	inv(7)(p15q34) t(7;7)(p15;q34) t(10;11)(p13;q14) t(11;19)(q23;p13) 9q34 deletion	HOXA HOXA CAML-AF10 MLL-ENL SET-NUP214	Frequent IL-7R mutation (38 %) Frequent (50 %) PHF6 mutations in SET-NUP214 Frequent RAS mutations in MLL-ENL CDKN2A deletion (67 %)	TCR αβ or TCR γδ Immature/ϒδ lineage Immature/ϒδ lineage Immature/ϒδ lineage
NKX2-1 and NKX2-2 (5 %)	inv(14)(q13q32.33) inv(14)(q11.2q13) t(7;14)(q34;q13) t(14;20)(q11;p11)	NKX2-1 (IGH) NKX2-1 (TRA) NKX2-2 (TRB) NKX2-2 (TRB)		Cortical thymocyte stage
MEF2 C (2 %)	del(5)(q14) der(5)t(4;5)(q26;q14)	MEF2C MEF2C (4q26)	NOTCH1/FBXW7 abnormalities (60 %) No mutations of PHF6 and WT1 PTEN/AKT mutations (13 %)	Early thymocyte progenitor

independently from the initial leukemic clone observed at diagnosis. In a second later study the same authors have explored a group of late-relapsing T-ALLs showing that there was evidence of: (a) a common clonal origin between diagnosis and relapse in 64 % of cases; (b) a different clonal origin between diagnosis and relapse in the remaining 36 % of cases (Szczepanski et al. 2011).

Mullighan et al. (2008a) studied copy number alterations in 14 matched T-ALL samples at diagnosis and at relapse, showing that the mean frequency of CNAs remained unchanged, but many of these alterations changed from diagnosis to relapse. A clonal relationship between the diagnosis and relapse T-ALL was observed in about 71 % of T-ALL cases; according to these findings it was suggested that the relapse CNAs either were present at diagnosis at low/very low levels and positively select at relapse or were acquired as new genetic alterations after the initial induction therapy. Tzoneva et al. (2013) have studied the mutational profile by using whole-exome sequencing of 5 T-ALL patients at diagnosis and at relapse: they identified a total of 60 mutations of whom 17 were present at diagnosis and at relapse, 24 were selectively present only at relapse and 19 were present only at diagnosis. About 80 % of these leukemias displayed at least one mutation at relapse, observed also at diagnosis; 20 % mutations all different at relapse, compared to those observed at diagnosis. The most relevant finding of whole-exome sequencing of relapsed T-ALL was the identification of mutations of the cytosolic 5′-nucleotidase II gene (NT5C2), encoding a 5′-nucleotidase enzyme responsible for the inactivation of nucleoside-analog chemotherapy drugs; NT5C2 mutant proteins display increased nucleotidase activity in vitro and confer resistance to 6-mercaptopurine and 6-thioguanine when expressed in T-lymphoblasts.

4 Leukemic Stem Cells in Acute Lymphoblastic Leukemias

4.1 B-ALLs

The nature and the frequencies of the stem cells or leukemia initiating cells in ALLs have been a contentious issue. In an initial study, Cobaleda et al. (2000) have shown that, regardless of the heterogeneity in maturation characteristics of the leukemic cells, only primitive blasts with a $CD34^{+}CD38^{-}$ immunophenotype were able to transfer BCR/ABL-positive ALLs into immunodeficient NOD/SCID mice. According to this finding, these cells were defined as SCID leukemia-initiating cells (SL-IC). Subsequently, it was proposed that an aberrant $CD19^{+}CD34^{+}$ lymphoid cell that lacks CD38 (the normal counterpart of this cell does not exist) expression could represent a candidate leukemic stem cell population in ALLs (Castor et al. 2005). This conclusion was based on the observation that clinically and genetically different subtypes of B-ALLs originate from different stages of hematopoietic differentiation: ETV6-RUNX1 (TEL-AML1) fusions-positive ALLs originated from committed B-cell progenitors, while major breakpoint BCR-ABL1 fusions (encoding P210 BCR-ABL1) originated at the level of HSCs (Castor et al. 2005). In

contrast, minor breakpoint BCR-ABL1 fusions (encoding P190 BCR-ABL1) had an origin at the level of cells with a B-cell progenitor phenotype (Castor et al. 2005). According to these findings it was proposed that P190 and P210 BCR-ABL1 were distinct tumor biological and clinical entities (Castor et al. 2005). In line with this observation, using samples of patients with ETV6-RUNX1 (TEL-AML) fusion, $CD34^{+}CD38^{low}CD19^{+}$ leukemic blasts were shown to be able to re-initiate and sustain leukemic growth in immunodeficient NOD/SCID mice. Particularly, using three samples from three patients with TEL/AML1-positive ALLs, only $CD34^{+}CD38^{low}CD19^{+}$ cells were able to engraft primary and secondary SCID mice, while transplantation of $CD34^{+}CD38^{-}CD19^{+}$ cells from only one of the three patients led to a low level of engraftment in primary, but not in secondary mice. Importantly, lentiviral transduction of normal cord blood progenitor cells with the ETV6-RUNX1 fusion gene led to the formation of cells with the aberrant $CD34^{+}CD38^{low}CD19^{+}$ immunophenotype (Hong et al. 2008). More recently, Cox et al. (2009) have provided evidence that in pediatric ALLs there is a minority (i.e., <1 %) of $CD133^{+}CD19^{-}$ cells that are capable of initiating and maintaining in vitro long-term cultures of B-ALL cells and of engrafting serial NOD/SCID recipient mice, with development of a B-ALL process; in contrast, there was no detectable engraftment with $CD133^{+}/CD19^{+}$ cells (Cox et al. 2009). At the level of the $CD133^{+}$ cell population, only $CD133^{+}/CD38^{-}$ cells were able to engraft immunodeficient mice.

The Philadelphia chromosome t(9;22) leading to the BCR/ABL fusion oncogene and the translocation t(4;11) with formation of the MLL/AF4 fusion oncogene have been associated with a particular poor outcome. Hotfilder et al. (2005) have analyzed 8 leukemic samples with ALL/t(9;22) and 12 with ALL/t(4;11) and have isolated immature $CD34^{+}CD19^{-}$ leukemic cells from these samples, showing by in situ hybridization that about 60 % of these cells carry the leukemic translocation. Through in vitro colony assays it was shown that myelo-erythroid colonies generated by $CD34^{+}CD19^{-}$ cells do not originate from a progenitor that carries the leukemic translocation. According to these findings it was concluded that childhood high-risk ALL/t(9;22) and t(4;11) originate in a primitive $CD34^{+}CD19^{-}$ progenitor/stem cell, without a myelo-erythroid developmental potential.

Subsequent studies have raised some doubts that ALL LSCs can be simply identified as $CD34^{+}CD38^{low}CD19^{+}$ cells. In fact, several studies have identified candidate LSCs in both rare, immature populations as well as conversely, across several immunophenotypically distinct groups of more mature cells (Cox et al. 2004; Kong et al. 2008; Le Viseur et al. 2008; Vormoor 2009). Particularly, it was shown that sorted $CD34^{+}CD19^{-}$, $CD34^{+}CD19^{+}$ and $CD34^{-}CD19^{+}$ cell populations all contain leukemia-initiating cells, although with different frequency. Importantly, each of these populations re-establish the complete immunophenotype of the original leukemia and is able so self-renew: this observation demonstrates the ability of B-ALL blasts to move back and forth between the different populations. It is important to note that in these studies the intrafemoral injection of sorted leukemic cells allowed a reproducible and efficient leukemic engraftment into the immunodeficient mice. Therefore, the intrafemoral injection of leukemic cells seems to be a robust transplantation assay to evaluate cell populations that are able to maintain ALLs in vivo.

It is also of interest to note that, in spite the efficient leukemic transplantation procedure developed in this study, not all ALL samples were able to engraft into NOD/SCID mice: particularly, while six out of seven high-risk ALLs were capable to grow into NOD/SCID mice, only two out six standard-risk patients engrafted (Le Viseur et al. 2008). Morisot et al. (2010) confirmed the preferential tendency of high-risk ALLs to grow in xenograft assay into highly immunodeficient mice: particularly, they observed that mice transplanted with primary samples from ALL patients at relapse developed leukemias in mice at 1–3 months post-transplantation, while those transplanted with primary samples from ALL patients at diagnosis developed leukemias more slowly, at 2–7 months post-transplant. Importantly, LSC frequency in precursor-B ALL was high, being evaluated in a range comprised between 1 % and 24 %. Other studies have shown that in primary childhood B-cell precursors both $CD34^+CD38^+CD19^+$ and $CD34^+CD38^-CD19^+$ cells exhibit an in vivo leukemogenic potential when grafted to a NOD/SCID mouse; in contrast, $CD34^+CD38^-CD19^-CD10^-$ cells do not generate a leukemic progeny in NOD/SCID mice, but a normal multilineage hematopoietic cell progeny (Kong et al. 2008). In a more recent study, Kong et al. (2014) have shown that $CD34^+CD38^-CD58^-$ cells are the leukemia-initiating cell population of Ph^+ ALLs. These studies were prompted by the observation that Ph^+ B-ALL patients with a predominant $CD34^+CD38^-CD58^-$ phenotype have a poorer prognosis than those with predominant $CD34^+CD38^+$ and/or $CD34^+CD58^+$ phenotypes. Importantly, only $CD34^+CD38^-CD58^-$, but not $CD34^+CD38^-CD58^+$ or $CD34^+CD38^+CD58^-$ or $CD34^+CD38^+CD58^+$ cells were able to engraft immunodeficient mice. The heterogeneity of immunophenotypic features of LICs in B-ALL was confirmed by Diamanti et al. (2012). In fact, these authors have shown that $CD34^+CD19^-$, $CD34^+CD19^+$ and $CD34^-$ cells isolated from B-cell precursor ALLs, all contain LICs.

An important property of the NOD/SCID model of ALL is its capacity to retain the genotypic and phenotypic properties of the original patient samples which provides a relatively accurate representation of the human disease. However, the NOD/SCID mice model possesses also some important intrinsic limitations and its capacity to be permissive for leukemic growth is certainly limited (Kennedy and Barabé 2008). These limitations are seemingly related to the lack of a supportive microenvironment or to the residual host's immune system preventing the engraftment of leukemic cells, blocking their capacity to reach tissual niches suitable for their survival and proliferation. In some recent studies new attempts have been made to develop new NOD/SCID transplantation assays based on the inoculation of leukemic cells in the spleen or in the liver. Thus, Wang et al. (2012) have reported the successful engraftment of ALL cells in NOD/SCID mice via intrasplenic inoculations: this assay implies the pre-treatment of mice with anti-CD122 mAb. This assay allowed the engraftment of ALL cells in 5 out 11 cases, with serial transplantation of the engrafted ALLs. Cheung et al. (2010) have reported the successful engraftment by ALL cells after direct intrahepatic injection into unconditioned newborn NOD/SCID mice. Five out 13 ALL samples engrafted into NOD/SCID mice using the intrahepatic route of leukemia cell injection.

A recent study provided interesting findings related to the frequency of LICs among different B-ALL subtypes, and in relationship with various immunophenotypic leukemic subpopulations (Rehe et al. 2013). It is important to point out that in this study were included pediatric B-ALLs pertaining to various B-ALL subtypes. Using these leukemic samples, it was investigated a possible relationship between membrane differentiation markers (CD34 expressed at the level of pro-B and pre-B1 lymphoid cells, CD10 expressed from pre-B1 to immature B-cells and CD20 expressed at low levels in preB-cells and at high levels in immature and mature B-cells) and LICs. The frequency of leukemia-initiating cells, as well as their kinetics of engraftment into immunodeficient mice, was comparable in leukemic blasts sorted according to the low/absent or high expression of either CD34, CD10 or CD20, thus indicating the absence of a link between leukemic cell differentiation status (as evaluated through the study of membrane markers) and LIC properties. Interestingly, the transcriptomic analysis of sorted $CD34^{+}$ and $CD34^{low/-}$ B-ALL cells showed a remarkable difference in their transcriptomic profile, with $CD34^{+}$ cells resembling normal B progenitors; however, in spite these differences in gene expression pattern, both these cell populations display a similar leukemia-initiating capacity (Rehe et al. 2013). A recent study directly addressed the problem of defining the stem cell program of purified populations of leukemic Stem/progenitor cells isolated from B-ALLs, compared to their normal counterpart. This type of analysis provided important data to define the stemness program of leukemic cells. To perform this analysis five populations of normal early lymphoid cells have been evaluated: HSC ($CD34^{+}CD38^{-}CD19^{-}$), Early Lymphoid Progenitor Cells (ELPC, $CD34^{+}CD38^{+}CD19^{-}$), Pro-B ($CD34^{+}CD38^{+}CD19^{+}$), Pre-B ($CD34^{-}CD19^{+}IgM^{-}$) and Immature/Mature B ($CD34^{-}CD19^{+}IgM^{+}$); these cell populations, at the HSC stage express "self-renewal" genes, including HOXB4, BMI1, TEL, AML1, PTEN, IKZF1, MLL and GFI1 and progressively acquire the expression of genes essential for B-cell development, such as, TCF3, EBF1, SPI1 and IKZF1 first, then DNTT, PAX5, VPREB1, RAG ½, LEF1 and IGLL1. On the other hand, four populations of leukemic cells have been isolated from TEL-AML1 B-ALLs: $CD34^{+}CD38^{-}CD19^{+}$, a leukemic-specific, early stem/progenitor cell population; $CD34^{+}CD38^{+}CD19^{+}$ operatively defined as ALL-Pro-B for its immunophenotypical similarity to normal Pro-B; $CD34^{-}CD38^{+}CD19^{+}$ defined as ALL Pre-B and, finally, $CD34^{-}CD38^{-}CD19^{+}$, defined as ALL-IM/M-B. All these four leukemic cell populations displayed a similar transcriptomic profile, independently on their phenotypic features and resembling all normal HSCs or ELPCs (Fan et al. 2014). This observation strongly supports the functional studies on isolated leukemic subpopulations showing that different immunophenotypical fractions of leukemic lymphoblasts contain LSCs.

The pattern of B-ALL growth into immunodeficient mice may reflect the leukemia prognosis. Thus, Meyer et al. (2011) have investigated the engraftment properties and impact on patient outcome of 50 pediatric B-ALL samples transplanted into NOD/SCID mice. Time to development of leukemia (TTL) into immunodeficient mice was determined for each patient sample engrafted as weeks from transplant to overt leukemia: accordingly, patients with a TTL <10 weeks were classified

as TTL^{short}, while those with prolonged time of NOD/SCID engraftment were classified as TTL^{long}. Importantly, patients whose leukemia samples exhibited TTL^{short} exhibited a clearly shorter survival compared to those with late leukemia onset. B-ALLs growing into NOD/SCID mice with a TTL^{short} pattern are associated with a gene expression signature characterized by high expression of signaling pathways involved in cell growth and apoptosis. These findings were confirmed and extended by the same authors in a subsequent study showing that an intact apoptosome function was associated with a TTL^{long} phenotype, good treatment response and better patient survival, while deficient apoptosome function was associated with rapid engraftment (TTL^{short} phenotype) and early relapse (Queudeville et al. 2012).

The differences observed between the different studies on the identification of leukemia-initiating cells in ALLs may be in part related to the intrinsic biologic heterogeneity of the leukemic stem cells observed in different B-ALL specimens, but are related also to technical differences in the methodology used to test in vivo the leukemia-initiating capacity of leukemic cell subpopulations. The assay-related variables are the following: (i) the NOD/SCID model (the NOD/SCID Gamma mice or NOD/SCID mice pre-treated with anti-NK lymphocytes lytic antibodies seem to be better recipients than the classical NOD/SCID mice); (ii) the site of leukemic cell injection into mice (the intrafemoral injection of candidate cells leads to a markedly more sensitive stem cell assay, compared to intravenous injection); (iii) the conditioning or not of recipient mice with irradiation. In line with this conclusion, a recent study provided important observations to optimize the experimental conditions for xenotransplant assays of human B-ALL samples. In fact, Patel et al. (2014) have explored a possible role of total body irradiation (TBI) pre-conditioning on the engraftment of human pediatric B-ALL cells into NSG mice. They observed that TBI preconditioning was associated with a markedly higher proportion of engrafting samples observed that TBI preconditioning was associated with a markedly higher proportion of engrafting samples (11/12), compared with no TBI (7/13). The analysis of B-ALL subtypes growing in the immunodeficient mice showed that while t(4;11) B-ALLs were able to grow efficiently also in unconditioned NSG recipient, the other B-ALL subtypes required TBI preconditioning for efficient engraftment into NSG mice. The superiority of the TBI preconditioning was apparent, not only when leukemic cells were injected IV, but also when leukemic cells were inoculated into bone marrow. The requirement for TBI preconditioning was related to the capacity of TBI to induce SDF-1 alpha release by bone marrow stromal cells and acting as a strong chemoattractant and homing factor for B-ALL progenitors.

Considering the ensemble of these studies one must conclude that the putative stem cells responsible for initiating and maintaining B-ALLs are not a fixed cell identity, but themselves evolve both in genotype and phenotype. This conclusion is supported by twin studies. These studies were based on the analysis of leukemic and pre-leukemic stem cell populations in the pair of identical twin discordant for ETV6-RUNX-positive ALL (Greaves and Wiemels 2003). As mentioned above, a subpopulation of $CD34^{+}CD38^{-}CD19^{+}$ cells was shown to be able to transfer the leukemia into NOD/SCID mice (Hong et al. 2008). These putative leukemic stem cells are present in both the healthy twin with pre-leukemia and in her co-twin with

ETV6-RUNX1-positive ALL: however, in the former one these cells are much less frequent than in the latter one. Importantly, pre-leukemic stem cells present in the healthy twin are genotypically and phenotypically distinct from leukemic stem cells observed in the twin with ETV6-RUNX1-positive ALL (Hong et al. 2008).

There is compelling evidence that several of the common translocations (i.e., MLL-AF4, TEL-AML1, BCR-ABL) that are seen in pediatric B-ALLs often originate prenatally in utero during embryonic/fetal development (Greaves and Wiemels 2003). The first evidence about the in utero origin of childhood B-ALLs is issued from studies in twins. In fact, leukemic cells isolated from identical twins with B-ALL share unique, specific, clonal chromosome rearrangements, a finding highly compatible with the hypothesis that these specific leukemogenic abnormalities derive from spontaneous mutagenic events occurring in utero (Ford et al. 1993; Wiemels et al. 1999). A second line of evidence indicates that during in utero development these leukemic fusion genes may arise in a population of mesodermal stem cells capable of differentiate during development in a variety of mesoderm-derived tissues, including Hematopoietic Stem Cells and Mesenchymal Stem Cells. This hypothesis was tested by investigating whether bone marrow-mesenchymal stem cells from childhood leukemia harbor leukemia-specific fusion genes. Mesenchymal Stem Cells of childhood B-ALLs carrying TEL-AML1 and BCR-ABL do not express the fusion transcripts; however, MLL-AF4 was detected and expressed in bone marrow-Mesenchymal Stem Cells from all cases of MLL-AF4-positive B-ALLs (Menendez et al. 2009). These observations indicate that MLL-AF4 arises in a population of mesodermal stem cells generating both hematopoietic and mesenchymal cells. Third, a prenatal origin of childhood ALLs was further supported by the detection of clonotypic immunoglobulin gene rearrangements on neonatal blot spots of children with various subtypes of ALLs (Greaves et al. 2003). Fourth, Teuffel et al. reported the results of a study carried out in 5-year-old monozygotic twins with concordant B-all displaying translocation of ETV6 and RUNX1 genes (ET6-RUNX1 fusion). Separate leukemic clones were identified in the diagnostic samples since distinct IGH and IGK gene rearrangements could be detected; importantly, both the identical ETV6-RUNX1 fusion sequence and the distinct immunoglobulin gene rearrangements were identified in the neonatal spots, thus unambiguously indicating that the separate leukemic clones evolved before birth (Teuffel et al. 2004). The study of twins with ALL was also of fundamental importance to determine the timing of mutation acquisition required for leukemia development. These studies were triggered by the observation that leukemic fusions are detectable in cord blood from healthy newborn infants at rates about 100 fold higher than the incidence of ALLs, thus suggesting a strict need for additional mutations in leukemia development (Mori et al. 2002).

According to these findings it was proposed a model suggesting that the ALL-specific fusion events occur in utero during embryonic/fetal development, generating a preleukemic clone, clinically silent; the preleukemic clone may progress to full leukemic transformation through the acquisition of new genetic abnormalities, such as point mutations, deletions and/or duplications. Thus, according to this model it is expected that TEL-AML1 fusion should occur in about 1 % of newborns,

taking into account the cumulative incidence of TEL-AML1$^+$ B-ALL in children of about 0.01 %. This expectation of incidence of TEL-AML1 fusion in newborns has been met by the study of Mori and coworkers reporting an incidence of about 1 % of TEL-AML1 among British-Italian newborns (Mori et al. 2002). These findings were confirmed by other investigators and, particularly, by Eguchi-Ishimal et al. (2002) showing that 1.5 % of tested cord bloods are positive for TEL-AML1 fusion. A Danish group has recently challenged this view, showing that the proportion of newborns with detectable TEL-AML1 fusion was lower (about 0.01 %), implying that a high proportion of infants born with detectable TEL-AML1 fusion develop TEL-AML1$^+$ B-ALL (Lausten-Thomsen et al. 2011). However, this was an isolated finding since the majority of other studies have shown high frequencies of TEL-AML1 fusions among newborns. Particularly, in the study led by Skorvaga et al. (2014), it was reported a frequency of 4 % of newborns exhibiting TEL-AML1 fusions. It is very important to point out that the TEL-AML1 transcripts are expressed in cord blood cells at very low levels, estimated as low as about one to five copies per 10^5 cells.

ETV6-RUNX1-positive ALLs, in addition to the fusion ETV6-RUNX1 gene, also have multiple copy number alterations (CNA), as revealed by genome-wide single-nucleotide polymorphism arrays: recurrent CNAs are seemingly driver events. The analysis of CNAs in five pairs of monozygotic twins with concordant ETV6-RUNX1-positive ALL showed that all the driver CNAs were discordant within each of the five twin pairs, thus suggesting that they are secondary to the prenatal gene fusion event (Bateman et al. 2010). In other studies the whole genomes of leukemic cells from some twin pairs with ALL have been sequenced, showing that few (5–10) shared prenatal coding-region single nucleotide variants were limited to the putative initiating lesions, while a relatively more abundant (15–20) non-synonymous single-nucleotide variants were distinct between tumors and, therefore, secondary and postnatal. These variants do not seem to affect genes relevant for the leukemogenic process, in agreement with the view that the leukemic development of ETV6-RUNX1-positive ALLs may be triggered by the initial fusion event and few CAN driver events (Ma et al. 2013).

The study of twins was also of fundamental importance to determine the timing and developmental sequence of molecular events in BCR-ABL1$^+$ ALL, usually associated with deletion of the IKAROS (IKZF1) gene. Through the analysis of the status of BCR-ABL1 and IKZF1 genes in some pairs of monozygotic twins concordant or discordant for Ph$^+$ ALL, it was reached the conclusion that the BCR-ABL1 is an initiation event occurring in utero, while the IKZF1 is a secondary and probably post-natal mutation. In the absence of the IKZF1 mutation, the leukemic clone remains clinically silent (Cazzaniga et al. 2011).

Similarly, there is evidence that MLL gene rearrangement with one of its fusion partner (AF4, ENL or AF9 genes) is an initiation, prenatal event. The penetrance of this genetic abnormality is very high and the concordance of ALLs in monozygotic twins bearing MLL rearrangements is near to 100 %. However, the study of rare cases of discordance of MLL-rearranged ALLs in monozygotic twins allowed to support the prenatal origin of MLL gene fusion event (Chuk et al. 2009).

Recent studies support the existence of genetic heterogeneity of leukemia initiating cells in ALLs. Using a multi-color, multi-plexed FISH method allowing the detection of the most recurrent or common genetic events occurring in a TEL-AML1 ALL (TEL-AML1 fusion, duplication of the fusion, extra copies of chromosome 21, deletion of unrearranged AML1 allele, mono- or bi-allelic deletions of PAX5 and CDKN2A/p16), it was possible to analyze individual stem cell clones in 30 patient's TEL-AML1 ALLs. This study provided evidence that leukemic stem cells in each patient are highly heterogeneous for their genetic alterations (Greaves 2009, 2010). These observations were definitely supported by a study carried out in ETV6-RUNX1-positive ALLs by multiplexing fluorescence in situ hybridization using the probes characterizing all known driver mutations occurring in this ALL subtype (TEL-AML1 fusion gene and few driver copy number alterations). This analysis allowed to define a composite picture of subclonal architecture, showing the existence of ALL subclones displaying a variegated genetics and complex evolutionary histories (Anderson et al. 2011). Leukemia-initiating cells are equally heterogeneous in the genetical abnormalities, showing a level of subclonal complexity highly similar to that observed in the bulk tumor cells (Anderson et al. 2011). Interestingly, the analysis of relapsing cases of ETV6-RUNX1-positive ALLs provided evidence that, irrespective of the time of relapse, the relapsing clone was derived from either a major or minor clone at presentation. Genetic events frequently observed in relapsing ETV6-RUNX1 ALLs are deletions of CDKN2A/B and gain of chromosome 16 (van Delft et al. 2011).

Ph^+ ALLs, rare in children (<5 % of pediatric ALLs) but frequent in adults (~35 % of adult ALLs), resemble CML lymphoid blast crisis and have a poor prognosis. The genetic lesions that cooperate with BCR-ABL to induce ALL have been in part characterized, including the frequent (>80 %) deletion of IK2F1 encoding the transcription factor Ikaros (Mullighan et al. 2008b), of PAX5 transcription factor (about 50 %) and of the inhibitors of cyclin D-dependent kinases CDKN2a/B (about 55 %) (Mullighan et al. 2008c). Taking advantage on the presence of frequent genetic abnormalities in Ph^+ ALLs attempts have been made to understand how the variability in these genetic abnormalities may reflect a genetic heterogeneity at the level of the leukemic stem cell compartment. Using various strains of NOD/SCID mice Notta et al. (2011b) have defined two subtypes of Ph^+ ALLs: one causing an aggressive disease in immunodeficient mice, the other inducing a non-aggressive leukemia in mice. The analysis of genetic lesions in these two subgroups showed: (a) similar frequencies of IKF1 deletions in the two groups; (b) marked differences in the frequencies of CDKN2A/B and PAX5 in the two groups (for CDKN2A/B 90 % in the aggressive group vs 0 % in the non-aggressive group; for PAX5 60 % in the aggressive group vs 10 % in the non-aggressive group) (Notta et al. 2011b). The analysis of clinical outcome showed a trend towards poorer outcome of aggressive patients with early relapse. By combining the xenografting and the DNA copy number alteration profiling it was provided evidence that genetic diversity occurs in functionally defined leukemia initiating cell subclones and that many patient samples contain multiple genetically distinct subclones. Reconstructing the subclonal evolution of leukemia-initiating cells of several ALL samples by copy number alteration

profiling allowed to support a branching multi-clonal evolution model of ALL leukemogenesis: for some patients, the predominant clone repopulated xenografts, whereas in other ones the predominant clone was competed by minor subclones. Reconstitution of xenografts with the predominant clone observed in the cells of the patients was associated with an aggressive growth in immunodeficient animals, a poorer patient outcome and the presence of additional mutations, particularly deletion of CDKN2A/B (Notta et al. 2011b).

The clonal architecture of MLL-AF4 infant B-ALLs was recently explored. As above mentioned, the MLL translocation with the AF4 partner gene is believed to be the initiating event occurring in utero. At variance with other B-ALLs, the disease development of MLL^+ ALLs does not seem to need additional, cooperating genetic abnormalities. However, although the copy number alterations are rare in MLL-AF4 patients at diagnosis, their number is more numerous at relapse, thus indicating genetic evolution of persisting MLL^+ leukemic clones (Bardini et al. 2010). Through the analysis of Ig/TCR rearrangement of MLL-AF4 at diagnosis and of xenograft leukemias derived the ALL samples, Bardini et al. (2014) have shown that MLL-AF4 ALLs are composed by a branching clonal and subclonal leukemia architecture, already at diagnosis; furthermore, investigation of paired leukemia samples at diagnosis and at relapse, indicated that relapse frequently occurs from clones pre-existing at diagnosis. Importantly, all the identified leukemic subclones are reflected at the level of leukemia-initiating cells, thus indicating that the cellular leukemic clonal/subclonal heterogeneity is dictated by a corresponding heterogeneity at the level of LSCs. Additional evidence in favor of clonally-related, but distinct subsets of leukemia-initiating cells was issued from the study of xenografts of high-risk precursor B-cell ALLs (Schmitz et al. 2011).

As above mentioned, TEL-AML1 confers a self-renewal advantage to stem cells. Some information are available about the mechanisms through which TEL-AML1 sustains stem cell growth and induces a growth advantage. Thus, it was shown that expression of TEL-AML1 in human cord blood progenitor cells led to expansion of a candidate preleukemia stem cell population with an early B phenotype ($CD34^+CD38^-CD19^+$) and a pronounced growth advantage in the presence of TGF-β (markedly reduced growth inhibition by TGF-β).

At the end of this section, it is important to mention new exciting therapeutic development obtained in the treatment of B-ALL with relapsing, refractory disease through CD19 targeting. As above shown, one of the antigens most frequently reported as expressed in B-ALL leukemia-initiating cells is CD19. This antigen is expressed during all stages of B-cell differentiation and this is expressed on the large majority of B-leukemic cells. The new therapeutic protocol consisted in the infusion of autologous T-lymphocytes transduced with CD19-directed chimeric antigen receptor lentiviral vector: through this procedure T-lymphocytes were redirected to address their cytotoxic activity to cells expressing CD19 (Mude et al. 2014; Lee et al. 2015). The advantage of this genetically engineered immunotherapy is double being mediated by cytotoxic T-lymphocytes highly efficient against $CD19^+$ cells and capable of long half-life in vivo and of tissutal trafficking. Recently, clinical data were made available about first B-ALL therapy treated with this new

approach. 90 % of the treated patients initially achieved a complete response. Of the patients who had a complete response 70 % remained in remission with an event-free survival rate of 67 % and overall survival rate of 78 % at 2 years (Mude et al. 2014). Taking into account the data on the CD19 expression of B-ALL leukemic stem cells, one could expect these findings and could speculate that only in a part of these patients displaying persistent complete remission the treatment could be curative (i.e., in those patients expressing CD19 at the level of all subpopulations of leukemia-initiating cells). It is important to note that these results are considerably better than those achieved using the best chemotherapy protocols for relapsed ALLs, allowing complete remission rates <25 % and median response duration <10 weeks.

4.2 T-ALLs

T-ALLs are about 15 % of all pediatric ALL cases. Difficulties in maintaining primary cultures of T-ALL cells and in developing in vivo models of T-ALL growth have limited for long time investigations into the biology of this malignancy. Studies carried out in these last years have in part elucidated the nature of the leukemia cells initiating T-ALLs. In this context, Cox et al. (2007) have sorted T-ALL cells for expression of CD34, CD4 and CD7: cells capable of in vitro and in vivo leukemic long-term growth were found among $CD34^+/CD7^-$, but not $CD34^+/CD4^+$ and $CD34^+/CD7^+$ (Cox et al. 2007). Importantly, in these experiments, 5×10^5 to 1×10^7 unsorted leukemia cells were required for engraftment, thus indicating that leukemia-initiating cells are rare in human T-ALLs.

In a subsequent study, Armstrong et al. (2009) have shown that the intrabone infusion of T-ALL blasts resulted in the constant engraftment of leukemic cells, with equally very high levels of engraftment into secondary and tertiary mice. The frequency of LICs into various T-ALL samples was variable and 10,000 leukemic cells were required to obtain engraftment into 100 % of immunodeficient animals. Importantly, in this study experimental conditions suitable for the maintaining of T-ALL LICs were determined, showing that co-culture of primary human T-ALL with a stromal line (MS5) expressing the NOTCH ligand delta-like-1 (DL1) reproducibly allowed to maintain T-ALL LICs and long-term growth of T-ALL cells. The sustained activation of the NOTCH signaling pathway into these cultures was strictly required for the survival and proliferation of leukemic cells: in fact, inhibition of the NOTCH pathway into primary cell cultures abolished in vitro cell growth of leukemic cells and in vivo T-LIC capacity.

Chiu et al. (2010) have used a stromal co-culture assay and NOD/SCID/IL-2Rγ^{null} (NSG) xenograft model using intrafemoral injection to characterize LICs from primary T-ALLs. Using this approach it was shown that $CD7^+CD1a^-$ cells isolated from primary T-ALL samples are responsive in vitro to proliferative signals mediated through NOTCH activation and are able to initiate leukemia into immunodeficient mice (Chiu et al. 2010). Expansion and clonal selection of leukemic cells generated by $CD7^+CD1a^-$ cells leads to the generation of a heterogeneous leukemic

cell population, with $CD7^+CD1a^+$ cells acquiring the property of LICs. Importantly, $CD7^+CD1a^-$ cells were shown to be resistant to glucocorticoid treatment and could be responsible for the development of drug-resistant T-ALLs.

Gerby et al. (2011) have fractionated T-ALL cells from primary leukemias into three cell fractions according to CD34 and CD7 positivity: the $CD34^+CD7^-$ fraction contained normal HSCs and HPCs; the $CD34^+CD7^+$ cell population was enriched in leukemia-initiating cells and proliferated in response to NOTCH activation and was inhibited by NOTCH inhibitors; $CD34^-CD7^+$ cell population contained more differentiated leukemic cells.

It is of interest to note that an optimal detection of leukemic stem cells in T-ALL samples requires fresh cells since the standard cryopreservation techniques determine a clear decrease of the frequency of these cells (Greystoke et al. 2013).

Interestingly, in some rare AML subtypes it was identified the existence of peculiar T-lymphocytic leukemia-initiating cells. These AMLs pertain to the group of AML samples unable to engraft into NOD/SCID mice (corresponding to about 40 % of total AMLs): about 30 % of these AMLs unable to grow into NOD/SCID mice are, however, capable of engrafting NOD/SCID/IL-2Rγ^{null} mice, but generated into these animals a monoclonal T-cell lymphoproliferative disorder similar to T-ALL. These grafts displayed self-renewal capacity as demonstrated by in vivo serial passages and their leukemia-propagating activity was restricted to $CD34^+$ cells. Molecular studies showed that these AML patient-derived LICs constantly expressed the MLL-AFX1 fusion product (Risueno et al. 2011).

Studies on the phenotype of a peculiar form of T-ALL, early T-cell precursor leukemia, suggest a peculiar origin of this T-ALL type. This type of T-ALL is characterized by an early T-cell precursor gene-expression signature and is associated with distinctive immunophenotypic features [$CD1a^-$, $CD8^-$, $CD5^{weak}$ with stem cell (CD34 and CD117) and myeloid (CD11b and CD13) markers] (Coustan-Smith et al. 2009). According to these findings it was suggested that this T-ALL is issued from the malignant transformation of early precursor T-cell, a subset of highly undifferentiated thymocytes representing immigrant T-cells from the bone marrow to the thymus, capable of multilineage differentiation (Bell and Bhandoola 2008; Wada et al. 2008).

As above mentioned, studies carried out in B-ALLs have shown a clonal heterogeneity at the level of both bulk leukemic cells and LICs. Similar evidence start to be obtained also for T-ALLs. In fact, Blackburn and coworkers, using a zebrafish transgenic model of T-ALL have obtained evidence about functional variation at the level of individual clones, with a minority of clones acquiring the capacity to activate AKT pathway and to increase their number of leukemia-propagating cells (Blackburn et al. 2014). These clones exhibited increased c-myc levels and are resistant to dexamethasone. According to these observations, it was suggested that T-ALL clones spontaneously and continuously evolve to leukemia progression through cellular mechanisms involving an increased frequency of LICs (Blackburn et al. 2014). The problem of tumor cell heterogeneity as a consequence of clonal LSC heterogeneity was specifically addressed by Clappier and coworkers. These authors have comparatively analyzed genetic lesions in T-cell ALL samples and in

xenograft derived from these samples: compared with paired diagnosis samples, the xenograft leukemias often contained additional genomic lesions occurring at the level of oncogenes and/or tumor suppressor genes and derive from minoritary subclones present in the patients at diagnosis. Furthermore, comparison of paired diagnosis and relapse samples showed that xenograft leukemias for their genetic abnormalities resembled more relapse samples than bulk diagnosis samples. Therefore, the establishment of T-ALL in immunodeficient mice is dependent on tumor cell heterogeneity existing in leukemic samples, selects and expands a more aggressive malignancy, recapitulating the leukemic progression and relapse of patients (Clappier et al. 2011).

5 Animal Models of Lymphoblastic Leukemias

5.1 Animal Models of T-ALL

5.1.1 NOTCH1

As above mentioned activating gain of function mutations in NOTCH1 have been observed in 50–70 % of patients with T-ALL. Initial studies carried out in animal models have shown that gain-of function NOTCH alleles that constitutively activate strong downstream signals are efficient inducers of leukemia in mice, while gain-of function NOTCH1 mutations commonly found in individuals with T-ALLs act as only weak tumor initiators (Chiang et al. 2008). However, these low, non-leukemogenic NOTCH1 mutants are able to complement other leukemogenic events, such as KRas activation (Chiang et al. 2008). Furthermore, NOTCH1 mutations have been identified in transgenic mouse T-ALL models driven by $KRas^{G12D}$ (Kindler et al. 2008). A NOTCH1 mutant, consisting of the transmembrane and intracellular domain of NOTCH1 (ICN1 mutant) was able to induce T-ALL in BM cells after transplant in mice (D'Altri et al. 2011).

As above indicated, PI3K-AKT pathway activation occurs in >85 % of T-ALL cases through various molecular mechanisms; activation of PI3K-AKT has been shown to collaborate with NOTCH1 in inducing leukemia development (Medyouf et al. 2010). The membrane IGF1R is an important target of NOTCH1 and its overexpression, frequently observed in T-ALLs, seems to represent one of the mechanisms through which Notch activation stimulate LICs in T-ALLs (Medyouf et al. 2012).

Calcineurin is a key determinant of Notch-mediated T leukemogenesis: in fact, calcineurin activation was found to be critical for leukemia initiating/propagating cell activity in T-ALL induced in mice by ICN1 NOTCH1 mutant (Gachet et al. 2013). Using a zebrafish T-ALL model, Blackburn et al. (2012) have reached the important conclusion that the primary role of NOTCH signaling in T-ALL development consists in the expansion of a population of pre-malignant early thymocytes and the acquisition of additional mutations by these cells is required for the full

transformation to leukemic progenitor cells. NOTCH1 cooperates also with ZMIZ1, a transcriptional coactivator of the protein inhibitor of activated STAT-like family, to induce T-ALL in mice. ZMIZ1 functionally interacts with NOTCH1 to promote c-MYC transcription and activity. ZMIZ1 inhibition slowed the growth and increased the sensitivity of tumor cells to NOTCH inhibitors (Rakowski et al. 2013).

The analysis of mouse models of NOTCH-induced T-ALL showed a differential effect of supraphysiological NOTCH signaling at the level of the leukemic and normal stem cell compartment: in fact, the enhanced NOTCH signaling promoted LSC activity in T-cell progenitors, but progressively extinguishes self-renewal of normal HSCs (Chiang et al. 2013). Other recent studies clearly showed that NOTCH1 expression at the level of human HSCs triggers T-cell differentiation as supported by studies based on the xenograft of human HSCs transduced with a constitutively active form of NOTCH1 (Haji et al. 2014).

The frequent occurrence of activating NOTCH1 mutations in T-ALLs and the key oncogenic role played by these mutations in leukemia development, have led to clinical trials evaluating the therapeutic effect of gamma-secretase inhibitors (GSI) that prevent NOTCH1 activation. However, the clinical responses to these drugs have been consistently limited in the time for the development of drug resistance. A recent study provided evidence that the resistance to GSI was due to the presence among naïve T-ALL cells of rare persister cells that through an epigenetic mechanism activate distinct signaling and transcriptional programs leading to drug resistance. The drug resistance of persister cells seems to be due to the expression of the transcription factor BRD4, essential for the viability of these cells and for the induction of c-myc and BCL2 expression in these cells. The essential role of BRD4 in mediating GSI-resistance of persister cells is supported by experiments carried out with the BRD4 inhibitor TQ1: this molecule induces growth arrest and apoptosis of persister cells (Knoechel et al. 2014).

5.1.2 FBXW7

FBXW7 is a constituent of the SCF (Sp1-Cul1-Fbox) ubiquitin ligase complex that controls the degradation and half-life of key proteins controlling fundamental cell pathways, such as Myc, NOTCH1, CyclinE and Mcl1. This gene is mutated in about 20 % of T-ALL patients: these mutations are usually heterozygous and cluster at the level of the substrate-binding domain. Monoallelic deletion of FBXW7 at the level of the hematopoietic system fail to induce leukemia; in contrast, complete FBXW7 deletion can lead to T-ALL development, but with low penetrance (Matsuoka et al. 2008). The development of a new generation of mice carrying Cre-inducible Fbxw7 heterozygous mutants, allowed to demonstrate that Fbxw7 deficiency does not affect HSC function and differentiation, but increases the number of leukemia-initiating cells; furthermore, Fbxw7 mutations cooperate with NOTCH1 mutations to induce T-ALL development in mice (King et al. 2013). The leukemia-promoting activity of Fbxw7 mutants correlated with their capacity to induce c-myc accumulation (King et al. 2013).

5.1.3 TAL1

TAL1, a transcription factor acting as a master regulator of hematopoiesis is mutated or translocated in about 25 % of childhood T-ALLs. TAL1 transgenic mice develop lymphomas with a mixed T and B-cell phenotype (Condorelli et al. 1996). Importantly, other studies have shown that transgenic mice expressing TAL 1 DNA binding mutants still develop T-cell leukemias/lymphomas (O'Neil et al. 2001). In line with these findings recent studies have shown that the oncogenetic role of TAL1 is played through its regulatory partners (including E2A, RUNX1, GATA3 and LMO1/2) through activation of MYB (Sanda et al. 2012) and microRNA-222 activation with consequent FBXW7 protein down-regulation (Mansour et al. 2013).

5.1.4 PTEN

One model was based on PTEN deletion in mouse hematopoietic cells that leads to a myeloproliferative disease, followed by T-ALL. In this model, PTEN inactivation in hematopoietic stem cells serves as a first hit to activate the PI3K-AKT pathway, conferring survival and proliferative advantages, and to promote genetic instability, leading to additional alterations: among them, the activation of beta-catenin may contribute to the acquisition of self-renewal capacity of leukemic stem cells, while t(14;15) chromosomal translocation results in T-lineage-specific overexpression of c-myc which may lead to T-ALL development (Guo et al. 2008). Therefore, the PTEN null model, with functionally defined populations of leukemic cells, one endowed with leukemia-initiating capacity ($CD3^{+}c\text{-}kit^{mid}$) and the other with blast properties ($CD3^{+}c\text{-}kit^{-}$), provides a unique opportunity to evaluate the effect of small molecule inhibitors on T-ALL development and, particularly, their capacity to target leukemic stem cells. Thus Schubbert et al. (2014) using this mouse model of T-ALL have shown that leukemia-initiating cells are targetable using combination therapy directed against the deregulated PI3K pathway and Myc. In both these models, an expansion of $c\text{-}kit^{+}CD3^{+}Lin^{-}$ cells is observed. Subsequent studies have shown that tumorigenesis in the context of a deficiency of PTEN in T-cell progenitors appears to be critically dependent on PI3Kγ and PI3Kδ isoforms (Subramanian et al. 2012).

5.1.5 IL-7R

The transduction of a mutant activating IL-7R into early thymocytes allowed to develop a model of human ETP-ALLs. In fact, in a recent study Treanor et al. (2014) showed that the transplantation of mouse early thymocytes $p19^{Arf-/-}$ transduced with a mutant IL-7R into recipient mice generated in vivo the formation of ETP-ALLs blocked at an early stage of differentiation, at which myeloid lineage and T-lymphoid differentiation programs co-exist.

5.1.6 LMO2

The cellular origin of T-ALL was investigated in a leukemia model of LMO2 oncogene activation. Particularly, to investigate the cellular origin of T-ALL, a cell mapping strategy was applied to a mouse T-ALL model to determine when the leukemia-initiating cell is established within the thymus. LMO2 transgenic mice express LMO2 in the thymus and develop T-cell leukemia similar to human T-ALL, after a latency of about 10 months. Analysis of the thymus cell populations during this time showed a preleukemic phenotype characterized by the accumulation of immature $CD4^{-}CD8^{-}$ thymocytes. These observations indicate that LMO2 promotes self-renewal of preleukemic thymocytes, providing a mechanism through which committed T-cells can accumulate additional genetic mutations required for leukemic transformation (McCormack et al. 2010). These findings were reinforced by an additional study showing that mice transgenic for TAL1 and LMO1 show an expanded population of primitive thymocyte progenitors inhibited in their terminal differentiation; these oncogenes provide a favorable context for the acquisition of activating NOTCH1 mutations and the emergence of self-renewing leukemia initiating cells (Tremblay et al. 2010). The model of T-ALL development induced by LMO2 in cooperation with TAL1 was further explored showing that T-ALL cells generated in these mice are heterogeneous and only 1 out 10,000 leukemic cells was able to generate a leukemic process after transplantation. The leukemia-initiating-capacity of these leukemias requires NOTCH1 signaling since it was inhibited by γ-secretase inhibitors (Tatarek et al. 2011). In addition to NOTCH1 also LYL1 was essential for mediating the leukemogenic activity of LMO2. LYL1, as well as TAL1, is required for the binding to DNA of LMO2. While TAL1 expression in thymocytes is dispensable for LMO2, LYL1 expression in thymocytes is strictly required for LMO2 leukemogenic activity, particularly for that concerns induction of self-renewal of thymocytes and of stem cell-like gene signature (McCormack et al. 2013).

The mouse models of LMO2-induced T-ALL have been explored to determine its effect at the level of thymocyte progenitors. These studies have shown that the most remarkable effect of LMO2 consist in blocking the differentiation of T-cell progenitor cells and in inducing a stem cell signature into these progenitors (Cleveland et al. 2013). Using transgenic mice with enforced expression of LMO2 in T-cells by the CD2 promoter/enhancer, it was provided evidence that LMO2 induces T-cell leukemia by two pathways: in one pathway there was coordinated activation of LYL1, HHEX and MYCN, while in the other pathway NOTCH1 target genes are activated. It is of interest to note that the gene activation pathway involving LYL1, HHEX and MYCN is commonly observed in early T-cell precursor ALLs. Conditional inactivation of HHEX in CD2-LMO2 transgenic mice clearly attenuated T-ALL development (Smith et al. 2014).

The capacity of LMO2 to induce selectively T-cell leukemias is impressively demonstrated by the gene therapy studies performed by gamma-retroviral gene transfer for severe combined immunodeficiency-X1 and showing the development of T-ALL in 4 out 20 patients treated due to integration of the retroviral vector 5′ of the LMO2 gene (Hacein-Bey-Abina et al. 2008).

5.1.7 ERG-Induced T-ALLs

The Ets-related gene (ERG) is an Ets-transcription factor required for hematopoietic stem cell development and maintenance-ERG is well expressed in HSCs and HPCs and its expression is lost during hematopoietic differentiation. ERG expression is down-regulated during early T-lymphopoiesis, being absent in T lymphopoiesis, being absent in T-lymphocytes; however, ERG expression is maintained in T-ALLs. In about 50 % of T-ALLs ERG is overexpressed and its overexpression is associated with a negative outcome of these leukemias (Baldus et al. 2006). Given these findings, the effects of ERG overexpression in hematopoietic cells have been explored. Thus, using either a vav promoter-driven ERG transgenic overexpression (Tsuzuki et al. 2011), or retroviral-mediated ERG overexpression in bone marrow transplant mice (Thoms et al. 2011), two studies have reported the development of ERG-induced T-cell leukemias. In one of these two studies it was shown also that ERG expression in T-ALL cells is mediated by the binding of TAL1, LMO2, LYL1 to an enhancer element present in the promoter of ERG gene (Thoms et al. 2011).

5.2 Animal Models of B-ALL

5.2.1 ETV6-RUNX1

Numerous attempts have been made to develop a mouse model of ETV6-RUNX1 B-ALL. Thus, various investigators have attempted to induce leukemia formation through retroviral transduction of bone marrow cells or fetal liver with ETV6-RUNX1 vectors: no incidence of leukemia was observed and only an increase of immature B-lymphoid cells was detected. Bernardin et al. (2002) reported a low frequency (2 out 9) of leukemia induction following enforced expression of ETV6-RUNX1 in mouse bone marrow cells; the efficiency of leukemia induction markedly increased (6 out 8) when ETV6-RunX1 was expressed into p16/p19-negative mouse bone marrow cells. Similarly, studies based on the transgenic mice model showed that the expression of ETV6-RUNX1 under control of the heavy chain immunoglobulin promoter failed to induce leukemia formation. For leukemia development a secondary genetic event is required, such as co-expression of other mutant genes or mutations induced by carcinogens or irradiation (van der Weyden et al. 2011; Li et al. 2013). Interestingly, it was shown that ETV6-RUNX1 renders prone to leukemia development (after mutagenesis) only when expressed at the level of HSCs, but not of lymphoid progenitor cells; in line with these findings, ETV6-RUNX1 increases the number of HSCs and maintain these cells in a quiescent state (Schindler et al. 2009).

In line with findings observed using murine bone marrow cells, also using human cord blood fractions enriched in HSC/HPCs or in B-cell progenitor cells and transduced with TEL-AML1 expression vectors, it was reached the conclusion that ETV6-RUNX1 was not competent to confer self-renewal ability on progenitor cells

and to initiate leukemogenesis (Fan et al. 2014). $Lin^{-}CD34^{+}CD38^{-}CD49f^{+}$ CB-cells, transduced with TEL-AML1 were able to induce preleukemia when injected into NOD/SCID mice (Fan et al. 2014).

5.2.2 E2A-HLF

The oncogenic fusion protein E2A-HLF is a chimeric transcription factor that arises from the t(17;19) translocation in children B-ALLs and is associated with a very poor outcome. Various animal models have been reported. Two initial transgenic models based on the expression of the fusion gene E2A-HLF under the control of the Eμ enhancer with the SV40 promoter provided evidence about a significant transforming activity with a variable proportion (20–60 %) of animals displaying the formation of lymphomas, mostly of the T-cell lineage.

A more recent study showed that the transduction of a murine stem cell retrovirus to induce E2A-HLF expression failed to induce leukemia development; however, when BM cells were transduced to express E2A-HLF together with Bcl-2 and transplanted, induce the formation of B-ALLs, resembling human B-ALLs (Smith et al. 2002).

Yamasaki et al. (2010) have used an inducible knock-in approach to induce E2A-HLF expression in hematopoietic cells; however, using this approach no leukemia formation was observed. Through insertional mutagenesis secondary events required for leukemia development have been identified: particularly, the Zfp521/ZNF521 gene was identified as a cooperative gene for E2A-HLF to develop B-ALL.

Other studies were focused to determine the essential role of LMO2 in mediating the oncogenic activity of the E2A-HLF fusion gene: in fact, it was shown that E2A-HLF induces LMO2 expression in primary B-ALL cells and this expression is essential for leukemic survival (Hirose et al. 2010); E2A-HLF was able to immortalize primary lymphoid progenitors and this effect is mimicked by induced expression in these cells of LMO2 and Bcl-2 (De Boer et al. 2011).

5.2.3 E2A-PBX1

The mechanisms of E2A-PBX1-mediated pre-B-cell transformation and the molecular nature of direct E2A-PBX1 target genes and pathways remain largely unknown. Initial attempts at modeling E2a-PBX1-driven human B-ALLs have been unsuccessful because caused myeloid leukemias, but not B-ALLs. A first model able to replicate in mice E2A-PBX1 B-ALL was based on the development of transgenic mice in which the expression of the fusion gene was under the control of lymphoid-specific Lck upstream sequence, Eμ enhancer and TCR Vβ promoter: mice developing leukemia at late times, died for a B-ALL or mixed lineage ALL. The long latency required for leukemia development was reduced by co-expression experiments with Hox gene overexpression by viral insertional mutagenesis (Bijl et al. 2005).

5.2.4 BCR-ABL

Various experimental approaches have been explored to try to develop a suitable model of BCR-ABL$^+$ B-ALL. Using a retroviral bone marrow transduction/transplantation model, both p190 and p210 induce a fatal myeloproliferative disease in recipients of transduced marrow. When donors are pre-treated with 5-FU, recipients of p190 or p210-transduced bone marrow develop a mixture of CML and B-lymphoid leukemia. P190 is more potent than p210 for induction of B-lymphoid leukemia (McLaughlin et al. 1989). BCR-ABL-induced B-cell leukemia requires 4–12 weeks for its development and involves only the B-lymphoid lineage: these findings imply that it originates at the level of a B-cell-restricted progenitor and requires the acquisition of additional genetic events for leukemia development (Li et al. 1999). Using this mouse ALL model it was possible to demonstrate the essential role of the PI3K pathway, and particularly of mTOR kinase, in the BCR-ABL1-mediated transformation of B lymphoid progenitors (Janes et al. 2010).

In order to establish an efficient model of BCR-ABL B-ALL, Williams et al. (2006) have first developed a strategy to obtain the immortalization and growth factor independence of these cells through p19^{Arf} knockout: importantly, these cells do not undergo apoptosis when transduced with BCR-ABL expression vectors. Introduction of Bcr-Abkl into p19^{Arf}-deficient bone marrow progenitors induces rapid ex vivo outgrowth of pre-B lymphoid cells and induces a highly aggressive form of B-ALL when inoculated into syngeneic mice. Virtually, all the pre-B-cells obtained through this procedure have leukemic potential, as supported by the observation that as low as 20 such cells when infused into healthy syngeneic mice induce a rapidly fatal, transplantable B-ALL.

A recent study identified in the mice the type of B-cell progenitor that seems to be more prone to leukemic transformation by BCR-ABL. Particularly, these studies have provided evidence that B-1 progenitors (i.e., those more responsive to IL-7 and the only ones responsive to TYSLP) when transduced with BCR-ABL initiate the leukemic process more rapidly than do BCR-ABL expressing B-2 progenitors (Montecino-Rodriguez et al. 2014).

5.2.5 BCR-ABL-Like ALLs

Ph-like ALLs represent a subgroup of high-risk B-ALLs characterized by a gene expression profile similar to Ph$^+$ ALLs, poor prognosis and recurrent CRLF2 rearrangements, JAK1/2 point mutations, JAK2 fusion genes and tyrosine kinase mutations. No animal genetic models of this ALL subtype have been reported. However, Maude et al. (2012) have reported the successful xenotransplantation of Ph-like ALL blasts into NSG mice (with 18/21 leukemia engraftment) and have used these xenografts to demonstrate the sensitivity of leukemic cells to targeting with JAK or mTOR inhibitors.

5.2.6 MLL-Rearranged ALLs

The development of animal models of MLL-rearranged ALLs has represented the object of intensive studies during these last years. Metzler et al. (2006) have used the invertor conditional technology to create a mouse model of MLL-AF4. Transgenic mice expressing this fusion gene invariably developed B-cell neoplasias, but of more mature phenotype than usually observed in pediatric B-ALLs.

Chen et al. (2006) have produced MLL-AF4 knock-in mice by homologous recombination in embryonic stem cells: these mice have an increased number of lymphoid and myeloid cells in hematopoietic tissues and after a prolonged latency developed hematologic malignancies, most frequently consisting in B-cell lymphomas. These observations have suggested that MLL-AF4 per se is not sufficient to induce the development of an overt malignancy and additional secondary mutations are required. Using a slightly different transgenic approach, Krivstov et al. (2008) provided evidence that the expression of a MLL-AF4 allele resulted in the development of AML, and less frequently of pre-B ALL.

In subsequent studies it was explored the in vivo transforming potential of both MLL-AF4 and of its fusion reciprocal AF4-MLL. Transplantation of purified preparations of progenitor/stem hematopoietic cells transduced with MLL-AF4 failed to induce leukemia development, while the transplantation of the cells transduced with AF4-MLL elicited the formation of proB-ALL, B/T biphenotypic leukemias or mixed lineage leukemia. According to these findings it was proposed that the t(4;11) leukemia is based on two oncoproteins, MLL-AF4 and its reciprocal AF4-MLL (Bursen et al. 2010).

In other studies attempts have been made to induce oncogenic transformation of either human $CD34^+$ cells (Montes et al. 2011) or human embryonic stem cells (Bueno et al. 2012) by transducing MLL-AF4 in these cells. In human $CD34^+$ cells MLL-AF4 expression enhanced the hematopoietic repopulating cell function and clonogenic potential, but failed to induce leukemia development (Montes et al. 2011). In embryonic stem cells, MLL-AF4 expression enhanced the hemogenic specification, but impaired further hematopoietic commitment in favor of an endothelial cell fate (Bueno et al. 2012).

Since FLT3 is highly expressed in MLL-$AF4^+$ pro-B ALLs, it seemed of particular interest to investigate a possible cooperation between MLL-AF4 and FLT3 in the transformation of human $CD34^+$ cells. However, the results of these studies showed that FLT3 activation was not sufficient to immortalize or transform MLL-AF4-expressing human $CD34^+$ stem/progenitor cells, thus suggesting the existence of alternative genetic and/or epigenetic cooperating oncogenic lesions (Montes et al. 2014). Similar experiments have been carried out in human ESCs showing that FLT3 activation cooperates with MLL-AF4 fusion protein to abrogate the hematopoietic specification of ESCs, but was unable to immortalize/transform ESC-derived hematopoietic cells, again suggesting the need for alternative genetic cooperating hits (Bueno et al. 2013).

5.2.7 Stat5b/Pax5

As above mentioned, somatic alterations of Pax5, a transcription factor acting downstream with respect to the transcription factors TCF3 and EBF1 to commit lymphoid progenitors to a B-cell fate, are frequent (up to 50 %) in the high-risk BCR-ABL1-positive and BCR-ABL1-like B-ALLs. Given the essential role of Pax5 in B-cell development, Pax5-deficient mice are arrested at the pro-B-cell stage in the bone marrow. Studies carried out in transgenic model of B-ALL driven by the expression of Stat5 constitutively active (Stat5-CA) in hematopoietic cell suggested a tumor suppressor role for Pax5: in fact, Stat5-CA mice usually develop B-ALLs with a long latency and low penetrance; this tumorigenic process is markedly accelerated by Pax5 heterozygosity (Heltemes-Harris et al. 2011). Interestingly, tumors arising in Stat5-CA; $Pax5^{+/-}$ mice invariably retain the WT Pax5 allele (Heltemes-Harris et al. 2011). More recently, this model was re-explored using transgenic RNAi to reversibly suppress endogenous Pax5 expression in the hematopoietic compartment of mice: restoring endogenous Pax5 expression in established B-ALLs triggers B-cell differentiation inducing durable disease remission. It is important to note that even brief Pax5 restoration in B-ALL cells was sufficient to cause rapid cell cycle exit and inhibition of their leukemia-initiating-capacity (Liu et al. 2014a).

Recent studies have reported the frequent (about 9 % of cases) occurrence of activating Stat5b mutations in T-ALL patients. These Stat5b-mutated T-ALLs are characterized by Bcl-X_L overexpression and by apparently absent chromosomic abnormalities (Kontro et al. 2014). Another recent study confirmed the frequent Stat5b mutations (6.3 % of cases) in T-ALL patients. In this study it was shown that Stat5b mutations occur in the phosphotyrosine binding pocket of Stat5b (N642H). Interestingly, in two patients studied at diagnosis and relapse it was shown that in one patient the Stat5b mutation was present only at diagnosis, while in the other patient the Stat5 mutation was at the heterozygous state at diagnosis and at the homozygous state at relapse. Stat5b-mutated T-ALLs exhibited a higher tendency at relapse than the Stat5b-WT T-ALLs (Baudapalli et al. 2014). At the biochemical level, the mutant Stat5b resulted in constitutive Stat5b phosphorylation, activation of Stat5 target genes and growth factor independent proliferation.

6 Conclusion

Tremendous progresses have been made in the understanding of the molecular abnormalities observed in ALLs. These information have been essential for a molecular classification of these diseases in subgroups, and for the identification of new therapeutic targets. Importantly, the identification of these various molecular abnormalities have provided precious molecular markers for the identification of tumor cell subpopulations and for the understanding of cellular and molecular dynamic during tumor development and progression.

The parallel development of studies on the identification and characterization of leukemic stem cells into these tumors and their integration with molecular studies has provided the basis for a consistent initial understanding of the early stages of ALL development, with identification of putative leukemia-initiating cells and for definition of their heterogeneity and changes during tumor development/progression. It is largely expected that these studies will contribute to an improvement in the efficacy of the therapy of ALLs, particularly through targeting of membrane antigens selectively or particularly expressed on leukemic stem cells.

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Part III
Novel Approaches for Targeting Cancer Stem Cells

Chapter 15
Targeting Key Stemness-Related Pathways in Human Cancers

Krysta M. Coyle, Margaret L. Thomas, Mohammad Sultan, and Paola Marcato

Abstract It is increasingly apparent that cancer stem cells (CSCs) play a substantial role in the response of human cancers to therapy. Indeed, the failure of mainstream chemotherapies to reduce the CSC burden may explain the high rates of tumor recurrence and metastasis. The development of new, anti-CSC agents is thus of great importance to reduce cancer-related mortality. One strategy to target CSCs focuses on their dependence on cell-signaling pathways, which differ from the majority of the tumor cells; these pathways include the embryonic Notch, Wingless-related (Wnt), and Hedgehog (Hh) pathways. Recently, there has been a surge in the development and clinical evaluation of targeted anti-Notch, anti-Wnt, and anti-Hh agents. Herein, we discuss the signaling paradigm for each of these pathways, identify druggable targets, and discuss selected pre-clinical and clinical findings with agents targeting each pathway. A number of natural molecules have shown some efficacy in inhibiting these stemness pathways. Importantly, we consider other disease-specific targeted agents to discuss roadblocks to the success of these anti-stemness agents – including financial considerations, the development of resistance, and on-target adverse effects. Novel clinical trial elements are required to adequately assess the success of these agents; however, the future for anti-CSC therapy is promising.

Keywords Cancer stem cells • Stemness pathways • Notch signaling • Wnt signaling • Hedgehog signaling • Druggable targets • Targeted therapy

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1 Introduction

1.1 Cancer Stem Cells and Stemness Pathways

There is mounting evidence that, regardless of the cell-of-origin, the dysregulated proliferation and differentiation observed in many cancer types represents a return to an earlier developmental stage. The dependence of cancer cells and cancer stem cells (CSCs) in particular, on self-renewal and multipotency make them reliant on a select few signaling pathways governing these characteristics. Indeed, the difference between cancerous and normal tissues has been characterized as dependent on the loss of stem-cell regulated homeostatic mechanisms which contribute to the maintenance of normal cell numbers (Tan et al. 2006). We will briefly discuss the reliance of CSCs on Notch, Wingless-related (Wnt), and Hedgehog (Hh) signaling before discussing drug targets to modulate these pathways.

1.2 Signaling Paradigm

A few pathways govern the development of entire organisms, including Notch, Wnt, Hh, receptor tyrosine kinase (RTK), Janus kinase/signal transducer and activator of transcription (Jak/STAT), and transforming growth factor beta (TGF-β) pathways. As a result, they must be highly specific and well organized. Barolo and Posakony (2002) identified important characteristics which define the signaling paradigm of these developmental pathways. First, these select pathways must be able to activate different or overlapping subsets of genes in various contexts. To facilitate this, pathways demonstrate activator insufficiency. Activation of the pathway is insufficient to activate transcription of all target genes with the same response element. This can be mediated by active repression of target genes in inappropriate signaling contexts. This requires the presence of *cis*-regulatory elements which bind repressors or additional activators. Alterations often exist in negative regulators of these signaling pathways in various types of cancer (Pece et al. 2004; Westhoff et al. 2009). Second, developmental pathways require the cooperation of tissue-specific or cell-type-specific activators (Barolo and Posakony 2002). Binding sites for these local activators are often located near the signal-activated promoters and are signal-independent. For example, transcription activation in the Notch pathway requires the "CBF-1, Suppressor of Hairless, Lag-2" (CSL) complex and the mastermind-like proteins (MAML1-3 in humans). An alternatively spliced form of CSL (CSL-TREX) was identified in acute myeloid leukemia (AML) and was associated with improved outcomes (Mansour et al. 2008). Alterations in the co-activator MAML have been identified in mucoepidermoid carcinomas via a chromosomal translocation disrupting the Notch pathway (Tonon et al. 2003). In human-papillomavirus (HPV)-induced cervical cancer, preliminary data has suggested that the E6 protein interacts with and interferes with MAML as a transcriptional co-activator in Notch signaling.

This provides a possible mechanism for the inhibition of epithelial differentiation in HPV-induced cervical cancer (Wu and Griffin 2004).

The final characteristic identified by Barolo and Posakony is default repression (Barolo and Posakony 2002). In the absence of signaling through these developmental pathways, transcription is repressed. Each pathway has unique DNA-binding co-repressors; however, they often share non-DNA-binding co-repressors [such as the silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) and nuclear receptor corepressor (N-Cor)]. A number of alterations in co-repressors have been described in various cancer types (Bosserhoff et al. 2001; Sheng et al. 2004; Tostar et al. 2005; Fernández-Majada et al. 2007; Scales and de Sauvage 2009; Phelps et al. 2009), suggesting that these co-repressors play a not-insignificant role in modulating the self-renewal and cell-fate decisions of malignant cells. The signaling paradigm described by Barolo and Posakony (2002) is important to understand how alterations in developmental signaling pathways contribute to the pathogenesis of cancer. Additionally, the three characteristics they have identified contribute to the selection of appropriate targets in the pharmacological modulation of signaling pathways.

2 Targeting Stem Cell Signaling Pathways

2.1 Identifying Druggable Targets in Signaling Pathways

The convoluted nature and extensive cross-talk between the Wnt, Hh and Notch pathways makes identifying appropriate druggable targets difficult. Gashaw et al. of Bayer Health set out a list of five characteristics to define actionable drug targets (Gashaw et al. 2011). These include ensuring that: (1) target has a role in disease; (2) the target is disease-specific; (3) the target is not uniformly expressed throughout the body; (4) there is a target- or disease-specific biomarker to monitor efficacy; and (5) prediction of side effects is minimal. Finally, targets are more favorable for drug development if they, or corresponding biomarkers, are easily assayed.

The stem cell signaling pathways culminate in transcriptional responses, often characterized by the transcriptional activation of target genes. Targeting these transcriptional responses can be difficult as drugs must pass through the nuclear membrane, and only small molecules which can diffuse through the membrane, or proteins which can be chaperoned, will enter the nucleus (Lusk et al. 2007). The transcriptional co-factors involved in these responses also have convoluted structures and lack deep binding sites for ideal drug targeting (Grivas and Papavassiliou 2012). Targeting upstream segments of these signaling pathways, such as ligand:receptor interactions or kinases usually lack sufficient specificity. The potential of these targets is further limited by the redundancy between pathways and general cross-talk.

In many cases, targeting stem cell signaling pathways will not be disease-specific, which leads to a number of on-target side effects. These adverse effects are sometimes dose-limiting and have led to the pursuit of alternate druggable targets.

One potential solution to this issue is the use of naturally-occurring molecules, as discussed in Sect. 2.2.

2.2 Targeted Molecules or Naturally Occurring Molecules?

Several important issues should be reflected upon when considering the costs and benefits of targeted therapies compared to naturally occurring molecules. The cost of targeted therapy development is often astronomical when considering the number of patients who will benefit (Kantarjian et al. 2013). Many of these drugs are tested in cancer patients who have exhausted all other means of treatment, resulting in minimal benefits to overall survival.

Targeted therapies will always be of benefit to cancers which display consistent and widespread oncogene addiction (such as Her2-amplified breast cancers and MET-overexpressing liver tumors). Gleevec (imatinib), the tyrosine kinase inhibitor, is one of the major successes of targeted therapy development and is used to treat chronic myelogenous leukemia (CML) and gastrointestinal stromal tumors. However, many drugs under development are beginning to focus on smaller and smaller subsets of patients, and many have idealized this narrowing focus as the future of personalized medicine. At an average cost of $1 billion USD for FDA-approved clinical drugs (Goozner 2004), it will confer an enormous, perhaps unsustainable, burden to those patients who are being targeted and their health insurance providers.

Since 2007, at least 12 natural products or derivatives have been approved for cancer therapy (Basmadjian et al. 2014). This is an indication of the reemergence of naturally occurring molecules in the pharmaceutical field. It is important to consider why natural molecules have been historically successful as anti-cancer therapeutics (e.g. etoposide, campothecin, paclitaxel, and rapamycin). Natural molecules have been described to occupy a different "area" of biochemical space than synthetic compounds (Ganesan 2008). They are subject to different restrictions in structure and are made up of different building blocks than synthetic molecules. The structural complexity of these molecules contributes to their specific interactions with targets, decreasing the possibility of dose-limiting side effects (Basmadjian et al. 2014). Notably, as the evolutionary purpose of these natural molecules is not as disease-modifying drugs, iterative alterations to their structures can improve their profile as pharmaceutical agents, such as the semi-synthetic paclitaxel analog, docetaxel (Ganesan 2008).

Drug development in the area of embryonic signaling pathways provides an opportunity to look at the benefits of both targeted therapies and natural molecules. Importantly, many cancers display aberrant signaling through the Notch, Wnt and Hh pathways; this suggests a possible benefit to many patients via treatment with signaling antagonists. A variety of targeted agents have been developed to each of these pathways, and are discussed in the following sections. Additionally, many existing medicinal agents (such as non-steroidal anti-inflammatory drugs) and natural molecules (such as resveratrol and curcumin) have been investigated for their modulation of Notch, Wnt, or Hh signaling. These agents will also be discussed.

3 Notch Signaling Pathway

3.1 The Notch Pathway and Druggable Targets

The Notch pathway is an intercellular communication pathway which is highly conserved among multicellular organisms (Egan et al. 1997). Notch facilitates the maintenance of an undifferentiated state in stem cells, participates in cell fate decisions, and can induce terminal differentiation.

The four Notch receptors (NOTCH1-4) are single-pass transmembrane proteins; the extracellular portion interacts with Delta-like ligands (DLLs) or Jagged ligands (JAGs) on nearby cells (Fig. 15.1). Upon receiving a signal via DLL or JAG, tumor necrosis factor-alpha-converting enzyme (TACE) or another ADAM protease

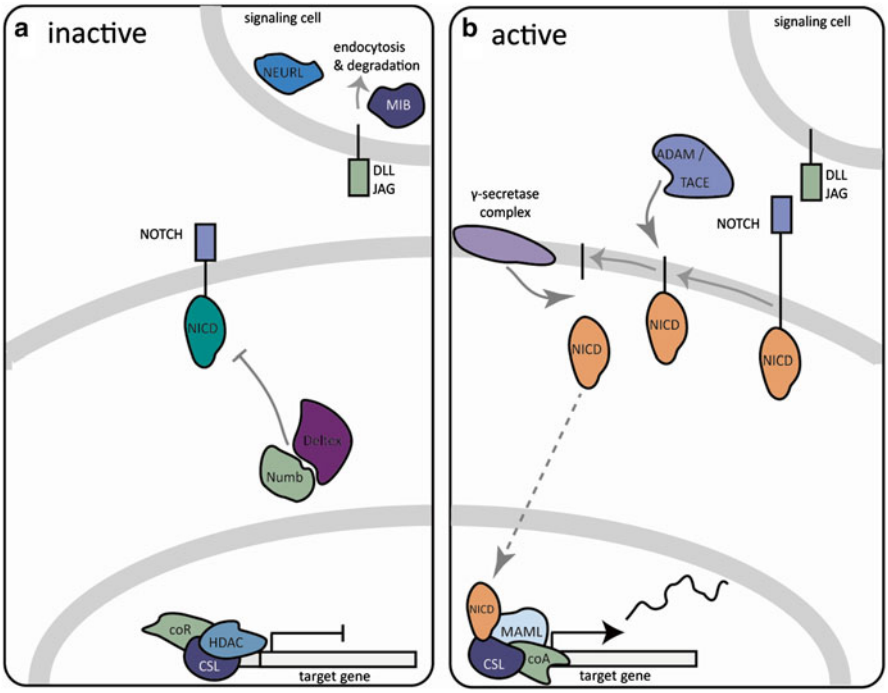

Fig. 15.1 Notch signaling results in transcriptional activation at target genes. (**a**) In its inactive state, DLL or Jagged ligands on signaling cells undergo endocytosis and degradation which is mediated by Neuralized (NEURL) and Mindbomb (MIB) ubiquitin ligases. Signaling from Notch intracellular domain (NICD) is inhibited by Numb and Deltex, and Notch-target genes are repressed by a combination of histone deacetylases (HDAC), other co-repressors (coR) and the CSL complex. (**b**) When Notch ligands bind to the Notch receptor, Notch undergoes a conformational change allowing cleavage of the extracellular domain by ADAM/TACE and subsequent cleavage of NICD by the γ-secretase complex. Following release, NICD translocates to the nucleus where it activates transcription in cooperation with Mastermind-like (MAML) and other co-activators (coA).

(that containing a disintegrin and a metalloprotease domain) cleaves the extracellular domain. This allows recognition of the Notch intracellular domain (NICD) by the y-secretase complex. The γ-secretase complex, consisting of nicastrin (NCSTN), presenilin (PSEN), presenilin enhancer 2 (PEN2), and anterior pharynx-defective 1 (APH1), releases the NICD from the transmembrane portion of the protein. NICD translocates to the nucleus, where it binds with the CSL complex to release co-repressors and recruit MAML and other co-activators. This activates transcription of Notch target genes, such as the Hes and Hey families of transcription factors.

The role of Notch signaling in oncogenesis is most clearly illustrated by T-cell acute lymphoblastic leukemia/lymphoma (T-ALL). Initially, Notch signaling was implicated in approximately 1 % of T-ALLs via the t(7;9)(q34;q34.3) chromosomal translocation. This translocation fuses the intracellular domain of Notch1 to the TCRβ promoter/enhancer, coupling T-cell development to constitutively activated Notch signaling (Reynolds et al. 1987). Two additional activating mutations were identified in Notch1, which occur in up to 60 % of T-ALL patients. The first of these leads to ligand-independent metalloproteases (ADAM/TACE) cleavage and release of the intracellular domain. The second stabilizes the intracellular domain and prevents its degradation.

While Notch-activating mutations are frequent in T-ALL, they have not been observed in other solid cancer types; this indicates that ligand-dependent activation predominates in activating aberrant Notch signaling (Roy et al. 2006). This activation of Notch signaling can be oncogenic in many contexts, resulting in increased invasion, migration, and proliferation. Oncogenic Notch signaling has been described in breast cancer, pancreatic cancer, glioblastoma, colon cancer, lymphoma and multiple myeloma (Stylianou et al. 2006; Wang et al. 2009; Li et al. 2011; Ylivinkka et al. 2013; Dai et al. 2014). Interestingly, there may be a specific role for Notch signaling in chemotherapeutic resistance and hypoxia-induced epithelial-to-mesenchymal transition (EMT) (Sahlgren et al. 2008; Wang et al. 2009).

Despite the multitude of evidence regarding the oncogenic role of Notch signaling, a number of groups have identified Notch as a tumor suppressor in several models (Sriuranpong et al. 2001; Nicolas et al. 2003; Proweller et al. 2006). Interestingly, Notch has been described as a tumor suppressor within the hematopoietic system, suggesting that the role of Notch is context specific, even within the hematopoietic system (Klinakis et al. 2011).

Identifying druggable targets in the Notch pathway is best done sequentially from extra-cellular-ligand binding through to activation of transcription at target genes (Fig. 15.1). First, preventing ligand:receptor interactions involves targeting the Notch receptor or the JAG/DLL ligands. Next, release of NICD, the intracellular molecule required for signaling activation, involves cleavage by ADAM/TACE and γ-secretase enzymes. Finally, transcription of target genes requires the CSL complex and MAML. A number of agents directed at these targets have been developed, and are in various stages of pre-clinical and clinical evaluation (Fig. 15.2). The most advanced agents are γ-secretase inhibitors, owing to overlap between Alzheimer's drug discovery and cancer therapy.

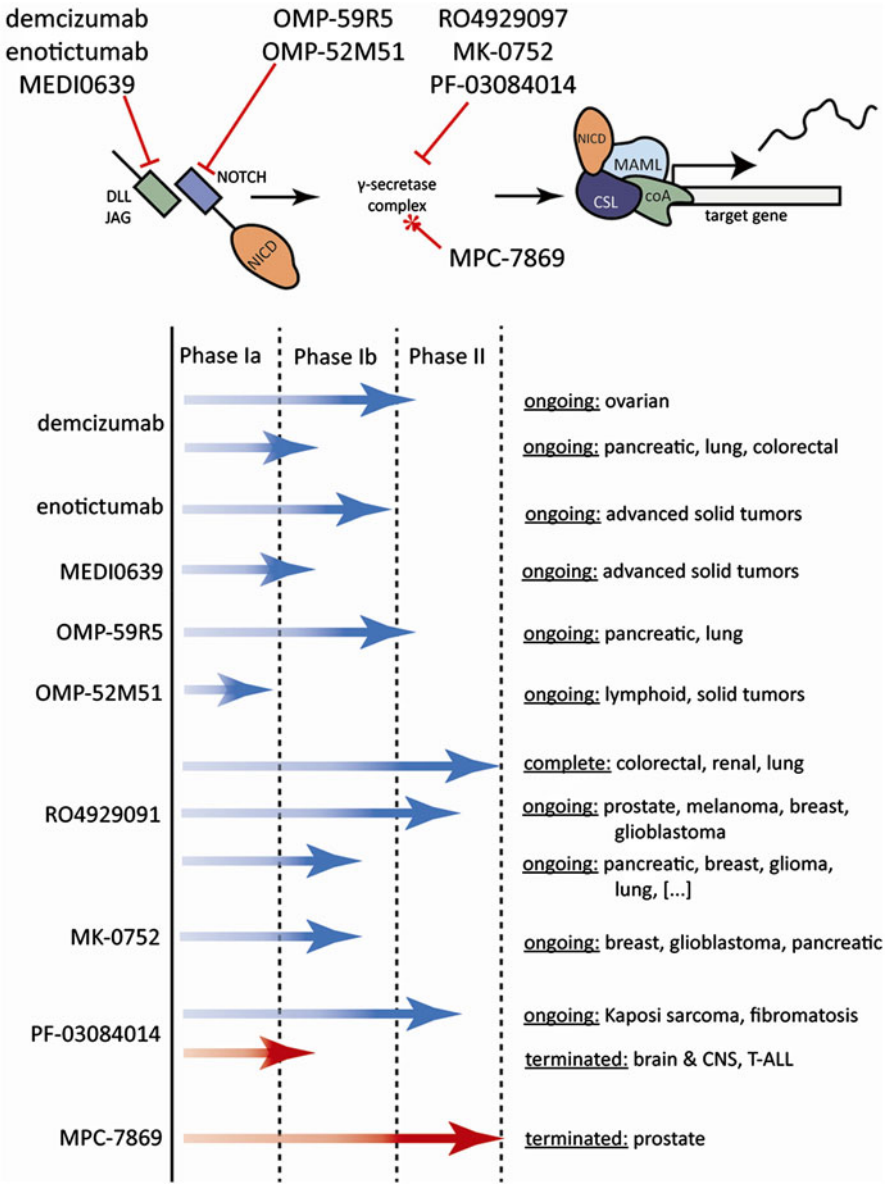

Fig. 15.2 Clinical trials of targeted anti-Notch agents have shown varying degrees of efficacy. A number of anti-DLL4 antibodies (e.g. demcizumab) and anti-Notch antibodies (e.g. OMP-59R5) have demonstrated promise in numerous cancer types. While GSIs are the most advanced in clinical development (e.g. RO4929091), they have not been as successful as those therapeutics inhibiting the Notch:ligand interaction.

3.2 Targeted Anti-notch Agents

3.2.1 DLL4 Monoclonal Antibodies

DLL4 is a Notch ligand which is also important for tumor angiogenesis. It is expressed by the tumor vasculature, and not often by the tumor cells. The expression of DLL4 in the vessels supplying the tumor seems to be regulated by VEGF, and expression levels of both DLL4 and VEGF correlate in tumors. The expression of DLL4 is low in the vasculature in normal tissues (Mailhos et al. 2002; Patel et al. 2006; Li et al. 2007; Jubb et al. 2009). Inhibition of DLL4-Notch signaling has led to increased vasculature; however, this is in general non-productive. This is due to hypersprouting of immature vessels, which are not able to perfuse the tissue efficiently (Thurston et al. 2007; Kuhnert et al. 2011). In fact, this non-productive angiogenesis inhibits tumor growth (Noguera-Troise et al. 2006). While DLL4 has a function in angiogenesis, DLL4-Notch signaling also plays an important role in CSC maintenance. Inhibition of DLL4 reduced CSC populations (Hoey et al. 2009). In colon cancer, inhibition of DLL4 leads to more differentiated colon cells (Hoey et al. 2009). However, targeting DLL4 is not without safety concerns. A study of chronic anti-DLL4 therapy identified changes in the livers of mice, rats, and cynomolgus monkeys; as well, skin lesions with features of vascular neoplasms were identified (Yan et al. 2010).

Demcizumab

In 2014, FDA granted Orphan Drug status for demcizumab (OMP-21M18, Fig. 15.2) in the treatment of pancreatic cancer. Early preclinical studies demonstrated that demcizumab inhibited expression of Notch target genes (Hoey et al. 2009). In combination with irinotecan, demcizumab decreased tumor growth and CSC frequency in a colorectal tumor model. A similar effect was seen when paclitaxel was combined with demcizumab in a breast tumor xenograft (Hoey et al. 2009). Preclinical studies in ovarian cancer xenografts demonstrated that demcizumab inhibited tumor growth and reduced CSC frequency (Yen et al. 2012). Treatment of pancreatic tumor xenografts with demcizumab also demonstrated the anti-tumor effects; interestingly, these effects were stronger when both human and mouse DLL4 were targeted (Yen et al. 2012). The most dangerous side effect observed in clinical studies (phase I) of demcizumab has been grade III asymptomatic hypertension in 28 % of patients. If anti-DLL4 treatment is to be combined with anti-VEGF therapy, patients must be carefully monitored (Ranpura et al. 2010; Twardowski et al. 2010).

Enoticumab (REGN421)

Enoticumab, a monoclonal anti-DLL4 antibody, is in phase I of development for advanced malignancies, led by Regeneron and Sanofi (Fig. 15.2). Preclinical treatment of ovarian tumor xenografts demonstrated an inhibition of tumor growth;

accompanied by an increase in tumor vascularization but reduced tumor perfusion (Kuhnert et al. 2013). These effects are consistent with those of other anti-DLL4 treatments. In a phase I study of patients with advanced solid tumors, several patients demonstrated prolonged stable disease or partial response (Jimeno et al. 2013a).

MEDI0639

The monoclonal antibody, MEDI0639 was identified by AstraZeneca as a specific, anti-DLL4 modulator of Notch signaling (Jenkins et al. 2012). Results of a safety study in cynomolgus monkeys identified a starting dose for a first-in-human phase 1 clinical trial; however, serious adverse events included reversible effects associated with gastrointestinal bleeding and heart failure (Ryan et al. 2013).

3.2.2 Notch-Targeted Antibodies

OMP-59R5 (Tarextumab)

Led by OncoMed Pharmaceuticals and GlaxoSmithKline, OMP-59R5 is an anti-Notch2/3 antibody in clinical testing (Fig. 15.2). Limited results are available from clinical studies. Phase I trials revealed dosages which were well-tolerated, and preliminary evidence of efficacy was observed (Spigel et al. 2014). Phase Ib and phase II proof-of-concept trials are ongoing in pancreatic cancer (with Abraxane® and gemcitabine) and in small cell lung cancer (with cisplatin and etopside).

OMP-52M51

OMP-52M51 is a humanized monoclonal anti-NOTCH1 antibody developed by OncoMed Pharmaceuticals (Fig. 15.2). Preclinical testing of OMP-52M51 in T-ALL demonstrated delayed tumorigenicity in samples from poor responders or relapsed patients (Agnusdei et al. 2013), and decreased CSC frequency in a xenograft model of breast cancer (Cancilla et al. 2013). Phase 1 single-agent trials are ongoing in hematologic and solid malignancies where NOTCH1 activation is implicated. Preliminary data from those with solid tumors demonstrates treatment was well tolerated (Davis et al. 2013).

3.2.3 γ-Secretase Inhibitors

Inhibitors of the γ-secretase complex, or GSIs, were initially developed to target the cleavage of the amyloid beta-protein precursor (AβPP) in Alzheimer's disease. Cleavage of AβPP by β- and γ-secretases generate the amyloid beta-peptide (Aβ) implicated in Alzheimer's disease. Treatment with GSIs in Alzheimer's clinical

trials identified a number of significant and serious side effects which have been attributed to the role of γ-secretases in Notch signaling throughout the body. These include an effect on the thymus, spleen and intestines (Wong et al. 2004; van Es et al. 2005; Demehri et al. 2009). A number of pre-clinical and clinical trials identified dose-limiting gastrointestinal side effects (Milano et al. 2004; van Es et al. 2005); however, combining GSIs with steroids, such as glucocorticoid or dexamethasone, has contributed to a decrease in these side effects (Real et al. 2008). These 'off-target' effects in the treatment of Alzheimer's disease lead to the investigation of these as 'on-target' effects in cancer therapy. Alarmingly, however, treatment with GSIs may increase the risk of skin cancer (Xia et al. 2001; Li et al. 2007; Demehri et al. 2009), suggesting that further characterization of patient tumors is required to determine the contexts in which Notch signaling is oncogenic or tumor-suppressive. A number of theoretical risks have also been suggested when considering GSIs as a cancer therapeutic, including damage to normal stem cells leading to goblet cell metaplasia (Searfoss et al. 2003; Wong et al. 2004). Drug discovery for Alzheimer's disease now focuses on modulators of γ-secretase activity, or Notch-sparing inhibitors; thus, there is no longer significant overlap between the cancer field and Alzheimer's field.

DAPT

N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), is a dipeptide non-transition state analog, specific γ-secretase inhibitor (Dovey et al. 2001). DAPT targets presenilin and prevents γ-secretase activity at a site distinct from the catalytic and substrate binding sites (Morohashi et al. 2006). In vitro, DAPT has been shown to deplete or inhibit CSC populations in nasopharyngeal carcinoma, lung carcinoma, metastatic breast cancer, and ovarian carcinoma (Jiang et al. 2011; McGowan et al. 2011; Yu et al. 2012; Liu et al. 2014). A number of other GSIs were developed from DAPT which are significantly more effective (e.g. RO-4929097, discussed below). It is thus not surprising that there are no clinical studies using DAPT.

L-685,458

An aspartyl protease transition state mimic, L-685,458 was identified in 2000 as a AβPP y-secretase inhibitor (Shearman et al. 2000). This GSI is not Notch-sparing and was demonstrated to block the colony forming ability of lymphoma CSCs by inhibiting the Notch pathway (Wang et al. 2011). In addition, inhibition of Notch by L-685,458 inhibited the growth of human tongue squamous cell carcinoma cells, accompanied by cell cycle arrest and apoptosis (Yao et al. 2007). L-685,458 has been observed to inhibit the activity of signal peptide peptidases (SPPs), a family of aspartyl proteases that is closely related to the γ-secretase complex; as such, any observations about the anti-tumor efficacy of L-685,458 cannot be assumed to be γ-secretase dependent (Weihofen et al. 2003).

RO4929097

Preclinical profiling of RO4929097 (Hoffman-La Roche, Fig. 15.2) demonstrated it was a very selective and potent inhibitor of γ-secretase activity and inhibited Notch signaling in vitro and in vivo (Luistro et al. 2009). RO4929097 was effective in reducing tumor growth of a number of xenograft models including pediatric models and melanomas; this was accompanied by a decrease in tumor initiating potential of melanoma (Huynh et al. 2011). Preclinical studies suggested intermittent dosing in clinical studies (Luistro et al. 2009). Interestingly, preclinical studies in inflammatory breast cancer (IBC) indicated that RO4929097 sensitized IBC to radiotherapy; however, mammosphere formation efficiency increased, contradicting previous evidence from the melanoma xenograft study (Debeb et al. 2012). Characterization of clinical CSC frequency will be required to determine the effects of RO4929097 on tumorigenicity and CSC number. Data from a phase I study with RO4929097 and cediranib in patients with advanced solid tumors suggested the combination was well tolerated and some evidence of antitumor efficacy was observed (Sahebjam et al. 2013). Similar results were observed by Diaz-Padilla et al. in advanced solid tumors and Tolcher et al. in refractory metastatic or locally advanced solid tumors (Tolcher et al. 2012; Diaz-Padilla et al. 2013). A phase II trial in refractory metastatic colorectal cancer revealed no antitumor efficacy and suggested it not be pursued as a monotherapy for this patient population (Strosberg et al. 2012). A phase II study in previously treated metastatic pancreatic adenocarcinoma was well tolerated and stable disease was achieved in 25 % of patients. Enrollment was halted after development of RO4929097 was discontinued (De Jesus-Acosta et al. 2014). A number of clinical trials with RO4949097 are in progress (Fig. 15.2); however, the majority of these trials are no longer recruiting patients. Ultimately, while RO4929097 may have some synergistic effects with existing chemotherapies, it is unlikely it will achieve success as a single agent.

MRK003 and MK0752

Merck and Co., Inc. have developed two sulfonamide-containing non-transition-state analog GSIs, MRK003 and its human analog MK0752 (Fig. 15.2). MRK003 has been tested in pre-clinical settings, and informed the use of MK0752 in clinical trials. In a mouse model of Her2-amplified breast cancer, where tumors contain a larger percentage of CSCs, treatment with MRK003 eliminated CSCs and initiated tumor regression. MRK003 also inhibited the survival and tumor-initiating capabilities of CSCs (Kondratyev et al. 2011). In a xenograft model of pancreatic cancer, MRK003 enhanced the anti-tumor effects of gemcitabine; up-regulation of B-cell receptor signaling and nuclear factor erythroid-derived 2-like 2 pathway correlated with the response of xenografts to the MRK003/gemcitabine regimen (Mizuma et al. 2012). In a patient-derived xenograft of uterine serous carcinoma, MRK003 enhanced the effect of paclitaxel and carboplatin therapy (Groeneweg et al. 2014b). Using platinum-resistant patient-derived xenografts of ovarian cancers, MRK003 in

combination with paclitaxel and carboplatin demonstrated anti-tumor effects greater than that of paclitaxel and carboplatin alone (Groeneweg et al. 2014a). Preclinical testing of MRK003 demonstrated a reduction of CSCs in breast cancer tumor xenograft models and an enhanced effect of docetaxel. Although several studies did not observe a strong effect of MRK003 (Watters et al. 2009; Efferson et al. 2010), it is likely that enhanced profiling of those cancers which do benefit will determine a previously-unidentified factor affecting the response of these tumors to MRK003 – and possibly to other GSIs. Clinically, the human analog, MK0752, in combination with docetaxel, resulted in a decrease of CSCs in patient tumors. Preliminary evidence of efficacy was observed, suggesting further clinical trials are warranted (Schott et al. 2013). Results from a phase I trial in pediatric patients with refractory central nervous system (CNS) tumors determined that MK0752 was well tolerated; however, no objective responses were observed. Interestingly, dose-limiting GI symptoms were not observed in this pediatric study (Fouladi et al. 2011). Results from a phase I trial in adult patients with advanced solid tumors suggested a clinical benefit to patients with high-grade gliomas (Krop et al. 2012). The range of effects seen following treatment with MK0752 demonstrates that further stratification of patients is warranted to isolate only those who will benefit.

PF-03084014

Pfizer has developed PF-03084014, a selective tetralin amino imidazole GSI (Fig. 15.2). A 2010 pre-clinical study determined that PF-03084014 reduced NICD levels and down-regulated the transcription of Notch target genes. The same study identified a dosing schedule which reduced gastrointestinal toxicity (Wei et al. 2010). In T-cell acute lymphoblastic leukemia (T-ALL), the combination of PF-03084014 with glucocorticoids contributed to a reduction of leukemic burden in a xenograft model (Samon et al. 2012). A pre-clinical study in breast cancer used docetaxel to activate the Notch pathway; subsequent treatment with PF-03084014 reversed these effects and synergistically induced tumor regression in a xenograft model (Zhang et al. 2013a). A combination of PF-03084014 and gemcitabine was effective at inducing tumor regression in a xenograft model of pancreatic ductal adenocarcinoma (PDAC) (Yabuuchi et al. 2013) and also reduced CSC ($CD24^{-}/CD44^{+}$ and $Aldefluor^{+}$) burden. PF-03084014 also demonstrated efficacy in colorectal xenografts with high activation of the Notch and Wnt pathways (Arcaroli et al. 2013); however, demonstrated limited efficacy as a single agent in pediatric xenograft models of solid and T-ALL tumors (Carol et al. 2014). We await the results of ongoing clinical trials to evaluate the efficacy of PF-03084014.

MPC-7869

The use of γ-secretase modulators (GSMs), such as MPC-7869 (tarenflurbil, Flurizan™), was intended to reduce the off-target effects of GSIs and minimize their dose-limiting toxicities. GSMs do not affect the rate of enzyme processing or

cause a build-up of substrates. MPC-7869 is based on the non-steroidal anti-inflammatory drug (NSAID) scaffold. Ultimately, MPC-7869 did not affect the γ-secretase cleavage of Notch, allowing signal transduction through the Notch pathway (Kukar and Golde 2008). After a double-blind, placebo-controlled clinical trial in prostate cancer failed to meet its efficacy endpoints (NCT00045123), Myriad Genetics Inc. discontinued its development as a cancer therapeutic (Fig. 15.2).

Conclusion

Current clinical trials of several GSIs are addressing the toxicity and efficacy of these drugs. Unfortunately, numerous mechanisms of resistance have been identified which will affect the success of GSIs in cancer therapy. One example is PTEN loss, which commonly occurs in T-ALL and contributes to GSI resistance (Palomero et al. 2008). Overexpression of MYC also contributes to GSI resistance (Rao et al. 2009). Cells which are resistant to GSIs demonstrate distinct signaling and transcriptional profiles, which have been attributed to a modified epigenetic status (Knoechel et al. 2014). Other mechanisms for GSI resistance have also been described (Watters et al. 2009; Wang et al. 2011; Miyamoto et al. 2013). Several of these mechanisms may be bypassed if GSIs are included with other classes of agents such as histone deacetylases (HDACs) or proteasome inhibitors, which have enhanced the effects of GSIs in T-All (Sanda et al. 2009). Complete pre-clinical testing is essential to rationalize the use of GSIs in various disease states (Tejada et al. 2014).

3.2.4 Other Agents

MAML-Stapled Peptide

MAML proteins are critical coactivators for the transcription of Notch-target genes, and have been implicated in the cross-talk with other signaling pathways such as Wnt/β-catenin (Alves-Guerra et al. 2007). As mentioned earlier, targeting nuclear proteins presents a significant difficulty for drug delivery. A 2006 study identified that a dominant-negative (dn) form of MAML functioned as a pan-Notch inhibitor (Proweller et al. 2006), and further investigations led to the development of a stapled fragment of dnMAML to prevent binding of its full-length, functional counterpart of the CSL complex. This prevents transcriptional activation of Notch-target genes. Preclinical testing of this model in GSI-sensitive T-ALL cell lines reduced the proliferation and leukemia-initiating capabilities of these cells (Moellering et al. 2009).

Anti-nicastrin Agents

In a pre-clinical study, silencing of nicastrin (a component of the γ-secretase complex) resulted in a decrease of breast cancer cell motility and invasion. Similar findings were observed with anti-nicastrin antibodies in vitro. The authors suggest

that a nicastrin-blocking antibody may be an effective therapy against metastasis of breast cancer (Filipović et al. 2014). Further in vitro testing as well as investigations in clinical settings will determine the efficacy of this strategy in other cancers.

3.3 Conclusion

GSIs remain the most advanced drugs targeting the Notch pathway. While GSIs have been associated with a number of side-effects including dose-limiting gastrointestinal toxicity and an increased risk of skin cancer, it is unclear whether the other Notch-targeting agents will have these same side effects. Further clinical testing will identify the consequences of chronic treatment using anti-DLL4 or anti-Notch antibodies.

4 Wnt Signaling Pathway

4.1 Wnt Signaling and Druggable Targets

The canonical Wnt signaling pathway functions in embryonic development and carcinogenesis by regulation of gene transcription. Wnt signaling is activated by the binding of a WNT ligand to the frizzled (FZD) receptor and low-density lipoprotein receptor-related protein (LRP) 5 or LRP6 on the cell surface. Dishevelled (DVL), adenomatous polyposis coli (APC), and axin are recruited to FZD, where they inhibit the activity of glycogen synthase kinase 3β (GSK3β) (Fig. 15.3). This promotes the stabilization of β-catenin, which enters the nucleus, binds to TCF/LEF transcription factors and activates the transcription of β-catenin target genes (e.g. c-myc, cyclin D, c-Jun, CTLA4). In the absence of WNT ligands, GSK3β phosphorylates β-catenin which leads to its degradation in the proteasome. The T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factor is bound to Groucho and HDACs, preventing the transcription of target genes.

Wnt signaling is a major contributor to oncogenesis of colorectal cancers. Mutations in APC and β-catenin frequently occur, leading to constitutive activation of the signaling pathway. In other cancers, dysfunctional Wnt signaling is often a result of irregular activation. Breast CSCs have displayed increased nuclear localization of β-catenin, suggesting highly active Wnt signaling in this population, and a number of agents which inhibit Wnt signaling also selectively inhibit the growth and tumorigenicity of CSCs (Gupta et al. 2009; Khramtsov et al. 2010). Wnt signaling is essential for the initiation of pancreatic cancer, and β-catenin is highly expressed in cisplatin-resistant lung cancer cells (Zhang et al. 2013b, Wang et al. 2014).

Inhibiting Wnt signaling can be done at many levels. First, it may be possible to prevent the secretion of Wnt ligands. Next, the interaction between WNT and FZD

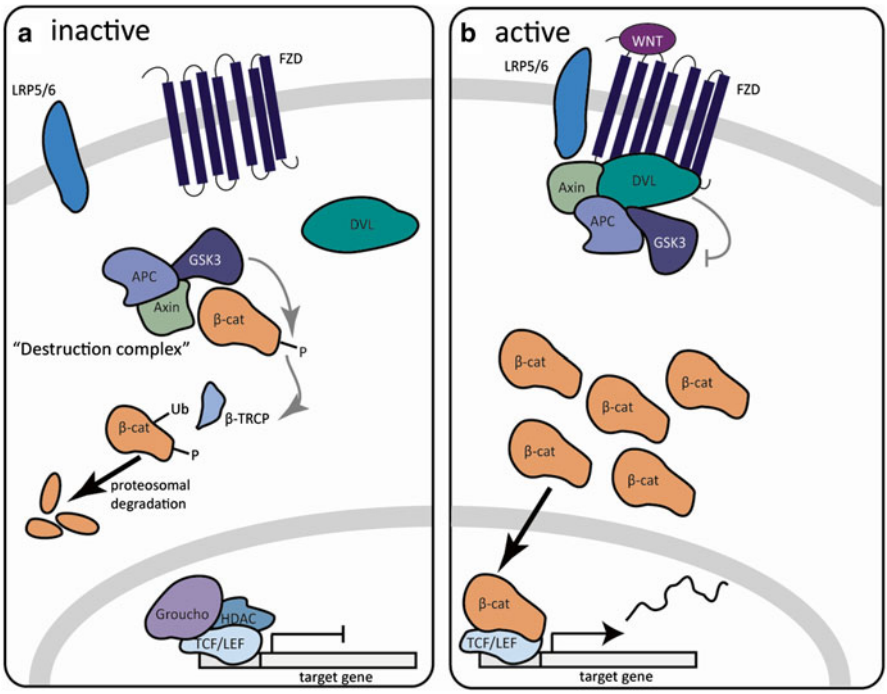

Fig. 15.3 Wnt signaling is dependent on the accumulation of β-catenin and its translocation to the nucleus. (**a**) In the absence of the WNT ligand, a "destruction complex" consisting of APC, GSK3β, and axin cooperate to phosphorylate β-catenin. This allows its ubiquitination, mediated by β-TRCP, and leads to proteasomal degradation. Wnt-target genes are inhibited by Groucho and HDAC binding to the TCF/LEF transcription factors. (**b**) When WNT ligands bind to the Frizzled receptor and LRP5/6, the "destruction complex" is recruited to Disheveled (DSH) at the membrane, inhibiting GSK3β. β-catenin is not phosphorylated and thus accumulates in the cytoplasm. The increasing levels of β-catenin drive it into the nucleus, where it can bind to TCF/LEF and activate transcription of target genes.

or LRP5/6 can prevent activation of downstream signaling. Finally, transcription of Wnt/β-catenin target genes can be prevented by antagonizing the binding of β-catenin to the TCF/LEF transcription factors or the CREB-binding protein (CBP) co-activator.

4.2 Targeted Anti-Wnt Agents

4.2.1 Porcupine Inhibitors

Porcupine (PORCN) is a membrane-bound O-acetyltransferase required for proper Wnt ligand secretion. Blocking Wnt ligand secretion by inhibiting porcupine activity may prevent full activation of the Wnt signaling pathway.

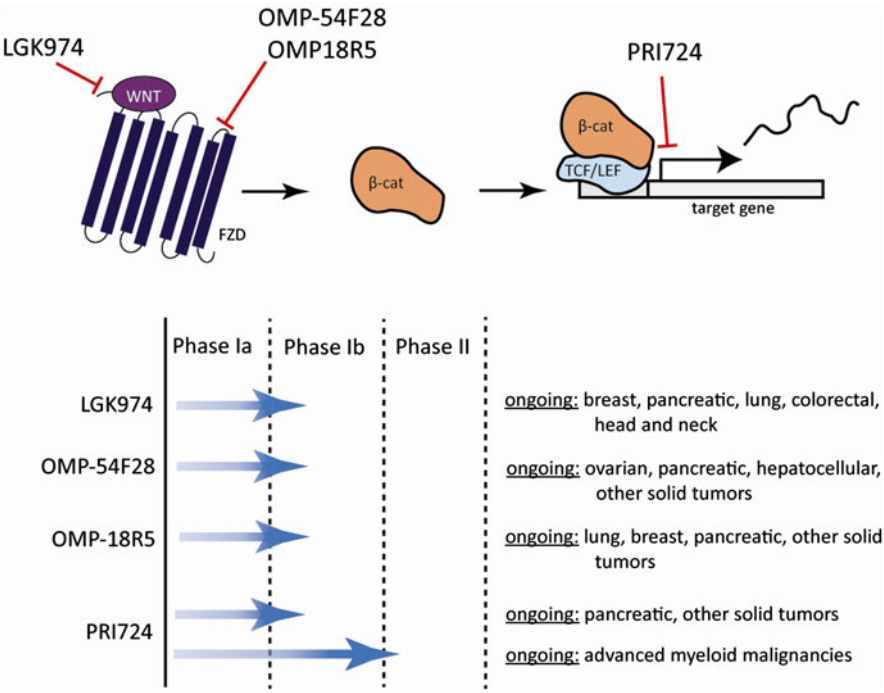

Fig. 15.4 Targeted anti-Wnt agents are early in clinical development. These clinical agents target a range of interactions in Wnt signaling. Molecules targeting WNT secretion (LGK974, PORCN inhibitor) or acting as Frizzled decoy receptors are in early phases of clinical testing, allowing minimal conclusions about their efficacy. PRI724, a CBP-inhibitor which antagonizes transcription of target genes, has a surprisingly low toxicity profile.

LGK974

A small-molecule screen led to the identification of LGK974 as a specific PORCN inhibitor by Liu et al. (Novartis, Fig. 15.4). They demonstrated its efficacy in murine models of Wnt-dependent breast cancer and human head-and-neck squamous cell carcinoma. Additionally, when used in combination with paclitaxel, it inhibited the growth of a human breast tumor xenograft (Liu et al. 2013). The results from an ongoing Phase I clinical trial will inform further use of this agent.

IWP Compounds

A cell-based synthetic-chemical screen identified several inhibitors of Wnt production (IWPs) as well as a number of inhibitors of Wnt response (IWRs). The IWP compounds, all sharing the same core chemical structure, specifically inhibited PORCN and subsequent secretion of Wnt ligands (Chen et al. 2009a). While IWP-2 has been tested pre-clinically in a number of models (Covey et al. 2012; Mo et al. 2013), its use as a clinical agent has not yet been determined.

4.2.2 Anti-frizzled Molecules

FZD8-Fc (Ipafricept)

The decoy receptor, FZD8-Fc (OMP-54F28, Fig. 15.4), consists of an immunoglobulin fragment-crystallizable (Fc) region fused to the cysteine-rich domain of FZD8 by a series of 8 amino acids. The minimal Fzd8 protein contains residues 1–155 and possible protease cleavage sites have been removed (DeAlmeida et al. 2007). This molecule binds Wnt ligands and prevents their signaling through native FZD receptors. Preclinical testing in an MMTV-Wnt1 tumor model as well as teratoma cell lines demonstrated significant anti-tumor activity accompanied by a decrease in expression of WNT-target genes (DeAlmeida et al. 2007). The FDA placed a partial clinical hold on ipafricept for 2 months (July–August 2014) due to observed on-target bone-related adverse events. Amendments have been incorporated into the ongoing Phase Ib clinical trial.

OMP-18R5 (Vantictumab)

Preclinical analysis of OMP-18R5, a monoclonal antibody (Fig. 15.4) which binds to five FZD receptors (FZD1, FZD2, FZD5, FZD7, FZD8), revealed anti-tumor effects on a range of tumor types including breast, NSCLC, pancreatic, colon, and teratocarcinoma; a decrease in tumorigenicity lowered to a decrease in CSC frequency (Gurney et al. 2012). Treatment of a mouse model of Kras-dependent pancreatic cancer with OMP-18R5 inhibited Wnt signaling and fewer pancreatic lesions were observed (Zhang et al. 2013b). Samples from patients enrolled in a phase Ia study of OMP-18R5 revealed that Wnt pathway target genes were regulated by vantictumab. There were dose-dependent effects on bone turnover markers (Smith et al. 2013). Increased bone turnover was observed, and more stringent exclusion criteria were developed in combination with prophylactic use of vitamin D and calcium, and use of zoledronic acid if required. Similar to the hold placed on ipafricept, the FDA placed a hold on vantictumab until amendments were made to phase Ib trials.

4.2.3 CREB-Binding Protein Targeted Agents

ICG-001

The small molecule ICG-001 binds CREB-binding protein (CBP) to disrupt its interaction with β-catenin and inhibit CBP function as a co-activator of Wnt/β-catenin-mediated transcription; however, its growth-inhibiting effects in PDAC cells were not due to inhibition of β-catenin-mediated transcription. Instead, microarray gene expression analyses implicated the potential disruption of DNA replication and cell cycle progression induced by CBP inhibition. Importantly, treatment

prolonged survival of PDAC-bearing mice, indicating the potential for CBP inhibition in PDAC treatment (Arensman et al. 2014).

PRI-724

Improvements to the ICG-001 structure led to the development of PRI-724. PRI724 is a specific CBP/beta-catenin antagonist with an extremely low toxicity profile (Fig. 15.4) (El-Khoueiry et al. 2013). This is somewhat surprising as CBP interacts with as many as 500 other cellular entities, including a large number of transcription factors (Lenz and Kahn 2014). Nevertheless, ongoing clinical trials will determine its efficacy as an anti-cancer agent.

4.3 Anti-Wnt Activity of Existing Medicinal Agents

4.3.1 Non-steroidal Anti-inflammatory Drugs

Non-steroidal anti-inflammatory drugs (NSAIDS) exert their anti-inflammatory, analgesic, and antipyretic effects by inhibiting cyclooxygenase (COX)-1 and COX2. An acetic-acid derivative NSAID, sulindac, and the COX2 inhibitor, celecoxib, have been shown to reduce ademonas in patients with familial adenomatous polyposis (FAP) (Huls et al. 2003). Patients with FAP commonly have inactivating mutations in APC, a negative regulator of Wnt signaling (Fig. 15.4). When NSAIDs are used in APC-mutant colorectal cells, Wnt signaling appears to be modulated (Stolfi et al. 2013); however, the precise mechanism of Wnt inhibition by NSAIDs is not fully understood. Some studies attribute the effects of NSAIDs to COX-dependent regulation of prostaglandin E2, which can suppress β-catenin degradation, while other studies have reported COX-independent mechanisms (Castellone et al. 2005; Buchanan and DuBois 2006). Understanding the mechanisms by which NSAIDs regulate Wnt signaling may lead to the derivation of new inhibitors which may have increased effectiveness as anti-cancer agents.

Acetaminophen

Wnt signaling is implicated in acetaminophen-induced liver injury (North et al. 2010), suggesting that acetaminophen may be able to modulate Wnt signaling at alternative dosages. Treatment of breast cancer cells in vitro with acetaminophen caused a decrease in β-catenin. The growth of subsequent engraftments of acetaminophen-treated cells was significantly impaired (Takehara et al. 2011).

Sulindac and Phosphosulindac

Sulindac binds to the PDZ domain (an interaction domain often found in scaffolding proteins) of DVL and blocks Wnt signaling (Lee et al. 2009). In patients treated with sulindac, nuclear β-catenin expression decreased from pre-treatment levels, suggesting a modulation of Wnt signaling (Boon et al. 2004). Sulindac treatment of colon cancer xenografts inhibited metastasis (Stein et al. 2011). Concomitant with a decrease in β-catenin levels, sulindac treatment inhibited proliferation of colon, lung, breast and prostate cancer cells (Han et al. 2008; Lu et al. 2008; Stein et al. 2011). Phosphosulindac, a safer and more effective derivative of sulindac, has been shown to inhibit the growth of breast and pancreatic cancer xenografts via inhibition of Wnt signaling and EMT in breast CSCs (Mackenzie et al. 2010, 2011; Zhu et al. 2012; Murray et al. 2013).

Celecoxib

The COX2 inhibitor, celecoxib, was approved by the FDA in 1999 for the treatment of FAP; however, this approval was withdrawn in 2011 as a decrease in colorectal cancer incidence upon treatment with celecoxib was not demonstrated. Treatment of colorectal cancer cells with celecoxib increases GSK3β kinase activity and phosphorylation of β-catenin. This was accompanied by a reduction of β-catenin/TCF dependent transcription (Sakoguchi-Okada et al. 2007; Tuynman et al. 2008). These effects have been attributed to the prostaglandin-E2 bioactive component of celecoxib (Castellone et al. 2005; Buchanan and DuBois 2006). However, a phase II trial of celecoxib in combination with gemcitabine and cisplatin in pancreatic cancer did not appear to have any benefit over the gemcitabine and cisplatin combination (El-Rayes et al. 2005). Selective targeting of tumors with high activation of Wnt signaling may be required to see any clinical benefit from celecoxib.

4.3.2 Antimicrobials

Streptonigrin

An antibiotic with anticancer activity, streptonigrin was investigated as early as 1967 (Smith et al. 1967). Treatment with streptonigrin has been demonstrated to inhibit proliferation of cancer cells with activated β-catenin/Wnt signaling. Streptonigrin treatment decreased nuclear β-catenin and β-catenin/TCF transcriptional activity. It is unclear whether this effect on transcription is a direct activity or whether it is due to suppression of upstream components such as GSK3β (Park and Chun 2011). Interestingly, a natural product screen determined that while streptonigrin was cytotoxic against melanoma cells, it was not effective against a CML cell line. Streptonigrin treatment also left a side-population of slow-cycling putative CSCs unaffected (Sztiller-Sikorska et al. 2014).

Salinomycin

The anti-CSC properties of salinomycin, an antibiotic potassium ionophore used in veterinary medicine, were first described in 2009 (Gupta et al. 2009). Salinomycin was isolated from *Streptomyces albus* in a soil sample from Japan (Naujokat and Steinhart 2012). Salinomycin has been demonstrated to down-regulate Wnt target genes in endometrial cancer cells (Kusunoki et al. 2013). This may be due to inhibition of phosphorylation of LRP6 (Lu et al. 2011a) or by activation of GSK3β and subsequent degradation of β-catenin (Tang et al. 2011; He et al. 2012; Wang et al. 2012). Evidence from breast cancer suggests that salinomycin is 100-fold more efficacious than paclitaxel at reducing the CSC frequency (Gupta et al. 2009). Unfortunately, salinomycin treatment has been associated with severe toxicity; a recent report attributes this to elevated cytosolic sodium levels, which subsequently increase cytosolic calcium levels, activating caspase 9 and 3 to reduce cell viability (Boehmerle and Endres 2011). Evidence from chronic lymphocytic leukemia suggests, however, that the effects of salinomycin on cell viability were specific to leukemic lymphocytes (Lu et al. 2011a). Safety evaluations and further pre-clinical testing will clarify the risk-to-benefit ratio of salinomycin.

Nigericin

Another potassium ionophore with a similar structure to that of salinomycin, nigericin, was observed to have anti-CSC characteristics (Gupta et al. 2009; Deng et al. 2013). Evidence has suggested that nigericin can inhibit the Wnt pathway, though the mechanism for this interaction is unclear (Lu et al. 2011a; Zhou et al. 2012).

Quinacrine

Wnt signaling can be inhibited by quinacrine, which up-regulates APC. This is followed by a subsequent decrease in activated GSK3β, and increased degradation of β-catenin (Preet et al. 2012). These effects have contributed to an inhibition of growth in breast cancer cells, while sparing normal breast epithelial cells (Preet et al. 2012).

Niclosamide

As an anti-helminthic, nicolasmide is used primarily in the treatment of tapeworms. Niclosamide blocks Wnt signaling in cancer cells via LRP6 degradation (Lu et al. 2011b). This induced apoptosis and inhibited proliferation of breast and prostate cancer cells. However, alternate evidence suggests that niclosamide antagonizes upstream Wnt signaling by promoting the endocytosis of FZD1 and down-regulating the DVL2 ligand (Chen et al. 2009b).

4.3.3 Other Agents

Tetrandrine

The calcium channel inhibitor, tentrandrine is a bis-benzylisoquinoline alkaloid purified from the root of *Stephania tetrandra*. In preclinical tests, tetrandrine exhibited better anticancer effects than 5-fluorouracil and carboplatin. In treated tumors, there was a decrease in β-catenin levels, suggesting that the anticancer activity of tetrandrine may be due to a modulation of Wnt signaling (He et al. 2010). The addition of tetrandrine enhanced the effects of cisplatin in cell line and xenograft models (Zhang et al. 2011b). One study suggested that tetrandrine specifically targets CSCs in breast cancer (Xu et al. 2012). In clinical testing, the addition of tetrandrine to a gemcitabine/cisplatin combination regimen in patients with advanced NSCLC improved short-term efficacy (Liu et al. 2012).

Trifluoperazine

The antipsychotic, trifluoperazine, inhibited the formation of tumorospheres in lung cancer models, which was accompanied by an inhibition of Wnt signaling. These effects enhanced the activity of gefitinib in animal models of lung cancer (Yeh et al. 2012). A network-based analysis suggests that these effects may also be observed when using other phenothiazine drugs such as chlorpromazine and fluphenazine (Qi and Ding 2013).

4.4 Conclusions

Of the three stemness pathways discussed in this chapter, it is intriguing that Wnt has been the focus of few targeted therapies. Instead, research has primarily focused on the use of natural products or existing medicinal agents in modulating Wnt signaling. It is unclear why this balance is different for Notch (Sect. 3) or Hh (Sect. 5). To date, some of the most successful pre-clinical findings in Wnt inhibition have been derived from natural molecules. While targeted therapies such as anti-FZD antibodies may reach an endpoint in their efficacy, developmental iterations of natural molecules will likely improve their efficacy.

5 Hedgehog Signaling Pathway

5.1 Hedgehog Pathway and Druggable Targets

The Hh signaling pathway functions in embryonic development and carcinogenesis by regulating gene transcription. The binding of a hedgehog ligand (Desert hedgehog DHH, Sonic hedgehog SHH, or Indian hedgehog IHH) to a 12-pass transmembrane

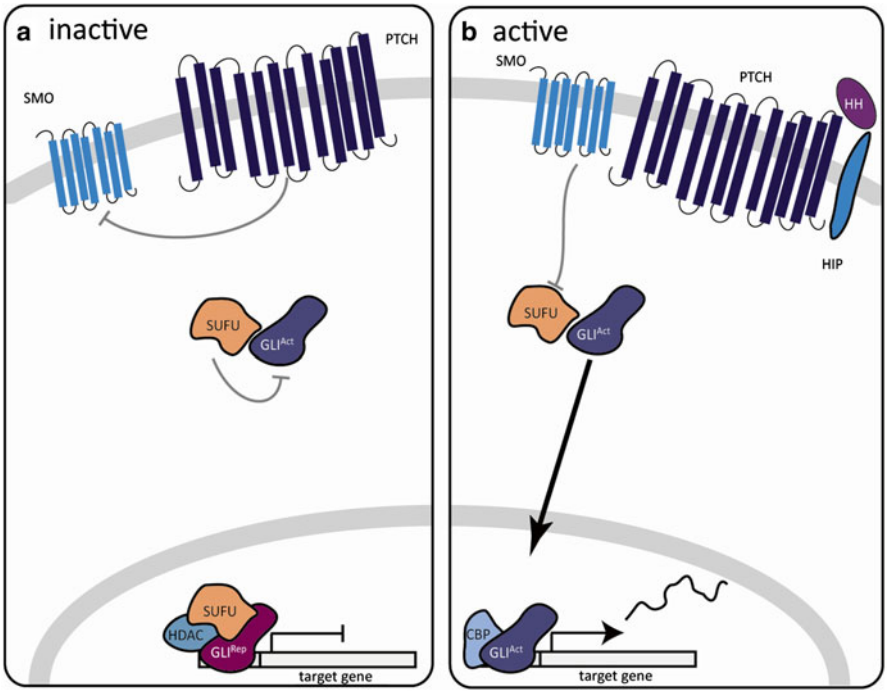

Fig. 15.5 Hedgehog signaling requires a balance between repressive and activating GLI proteins. (**a**) Endogenous Patched inhibits Smoothened, preventing its interactions with Sufu. Active Sufu inhibits activating GLI proteins, allowing repressive GLI to bind to Hh-target genes. When HH ligands bind to PTCH, the inhibition of Sufu is relieved, allowing activating GLI proteins to bind to CBP at target genes, activating transcription.

patched (PTCH) protein triggers the reversal of suppressor-of-fused (SUFU) inhibition of activating GLI proteins. The GLI proteins are effectors of Hh signaling and enter the nucleus, initiating a transcriptional response with CBP/p300 at Hh target genes (Fig. 15.5).

Hh signaling has been unambiguously linked to a particular subtype of medulloblastoma. Hh signaling regulates cerebellar patterning, linking mutations in pathway components such as PTCH or SUFU to the development of malignant brain tumors such as medulloblastoma. Approximately 30 % of medulloblastomas can be characterized by dysregulated Hh signaling (Northcott et al. 2012). Other cancers display activated Hh signaling, though to a lesser extent. For example, breast CSCs have higher expression of PTCH and GLI proteins compared to the non-CSCs (Liu et al. 2006; Shipitsin et al. 2007).

Important druggable interactions in the signaling pathway are the binding of HH ligands to PTCH, the PTCH: SMO interaction, and the GLI-mediated transcriptional response. In some cases, activation of Hh is downstream from SMO and these drug

candidates will not be effective (Nagao-Kitamoto et al. 2014). Thus, it is important to target downstream interactions such as GLI-mediated transcription.

5.2 Targeted Anti-Hedgehog Agents

5.2.1 Hedgehog: PTCH Inhibitors

5E1

This Hh pathway antagonist has been used in vitro and in vivo to study Hh signaling. 5E1, a monoclonal antibody, blocks binding of the Hh ligands to PTCH. In hepatocellular carcinoma cells with activated Hh signaling, 5E1 decreased expression of Hh target genes, inhibited cell growth and resulted in apoptosis (Huang et al. 2006). Xenograft growth of colorectal cancer cells and pancreatic was significantly decreased upon treatment with 5E1 (Yauch et al. 2008; Bailey et al. 2009). It has not progressed to clinical trials.

Robotnikinin

A high-throughput screen of aminoalcohol-derived macrocycles identified robotnikinin as a small molecule which binds the SHH ligand and prevents its interactions with PTCH (Stanton et al. 2009; Peng et al. 2009). A number of analogues were identified in a 2012 publication; however, none of these molecules have progressed to clinical trials (Dockendorff et al. 2012).

5.2.2 Smoothened Inhibitors

Cyclopamine

Sheep grazing on corn lily (*Veratrum californicum*) on a farm in Idaho gave birth to lambs with cyclopia, or one-eyed lambs. Cyclopamine and jervine were finally identified as the teratogenic components of the corn lily. It was not until the 1990s that the defects observed in these lambs were associated with dysregulated Hh signaling (Chiang et al. 1996; Cooper et al. 1998). Cyclopamine is a steroidal jervetraum alkaloid which binds SMO to inhibit Hh signaling (Chen et al. 2002). The mechanism of action of cyclopamine is not fully understood; however, it likely influences the balance between the active and inactive forms of SMO (Taipale et al. 2000; Chen et al. 2002). Cyclopamine, however, exhibits poor solubility, acid sensitivity, and weak potency when compared to other small-molecule antagonists. As such, derivatives of cyclopamine have been identified which have increased bioavailability and are more potent against human cancers (Zhang et al. 2008; Tremblay et al. 2008). One such derivative, IPI-926, is discussed below.

GDC-0449 (Vismodegib)

Development of GDC-0449, a small molecule of the 2-arylpyridine class (Genentech Inc. and Curis Inc., Fig. 15.6), was approved in 2012 by the FDA for treatment of metastatic or locally advanced basal cell carcinoma (BCC). Locally advanced BCC includes those patients with post-surgical recurrent tumors, and patients who are not candidates for surgery or radiation. While vismodegib is an important addition to the treatment options for those with locally advanced BCC, phase II evidence leading to the approval of vismodegib for locally advanced BCC consisted of a small number of patients in a single-arm study (Lyons et al. 2014). A 2012 report identified a novel phenomenon of BCC tumor regrowth in or near to the original vismodegib-sensitive tumor bed while therapy is ongoing. The mechanism for this is not clear and may be due to heterogeneity of the original tumor (Chang and Oro 2012). Further evidence and long-term follow-up data will be essentially to fully evaluate the efficacy of vismodegib in BCC and the benefit to patient survival.

Vismodegib is also under investigation in tumors of other origins. A phase I study determined that vismodegib was well tolerated in pediatric medulloblastoma patients (Gajjar et al. 2013). A phase II trial in metastatic colorectal cancer identified no benefit from vismodegib, and actually described lower treatment intensity for the other standard-of-care components. The authors suggest that toxicity may have contributed to this decreased efficacy (Berlin et al. 2012). A phase II trial in patients with ovarian cancer in second or third complete remission did not meet expectations for increased progression-free survival (Kaye et al. 2012).

BMS-833923

Bristol-Myers Squibb Co. and Exelixis Inc. have developed BMS-833923 (XL-139, Fig. 15.6). Treatment with BMS-833923 inhibited transcription of Hh target genes in esophageal adenocarcinoma cells and induced apoptosis (Zaidi et al. 2013). A phase I study of BMS-833923 demonstrated a partial response in one patient with basal cell nevoid syndrome with a known mutation in PTCH1 (Siu et al. 2009). Treatment was well-tolerated. The results from ongoing clinical trials will define its use as an anti-cancer agent.

PF-04449913

The identification of PF-04449913 was described by Munchhof et al. (Munchhof et al. 2011). Treating with PF-04449913 decreased tumorigenicity and leukemia-initiating potential of AML cells (Fukushima et al. 2013). The numerous clinical trials ongoing with PF-04449913 will instruct its future use in various cancer types (Fig 15.6).

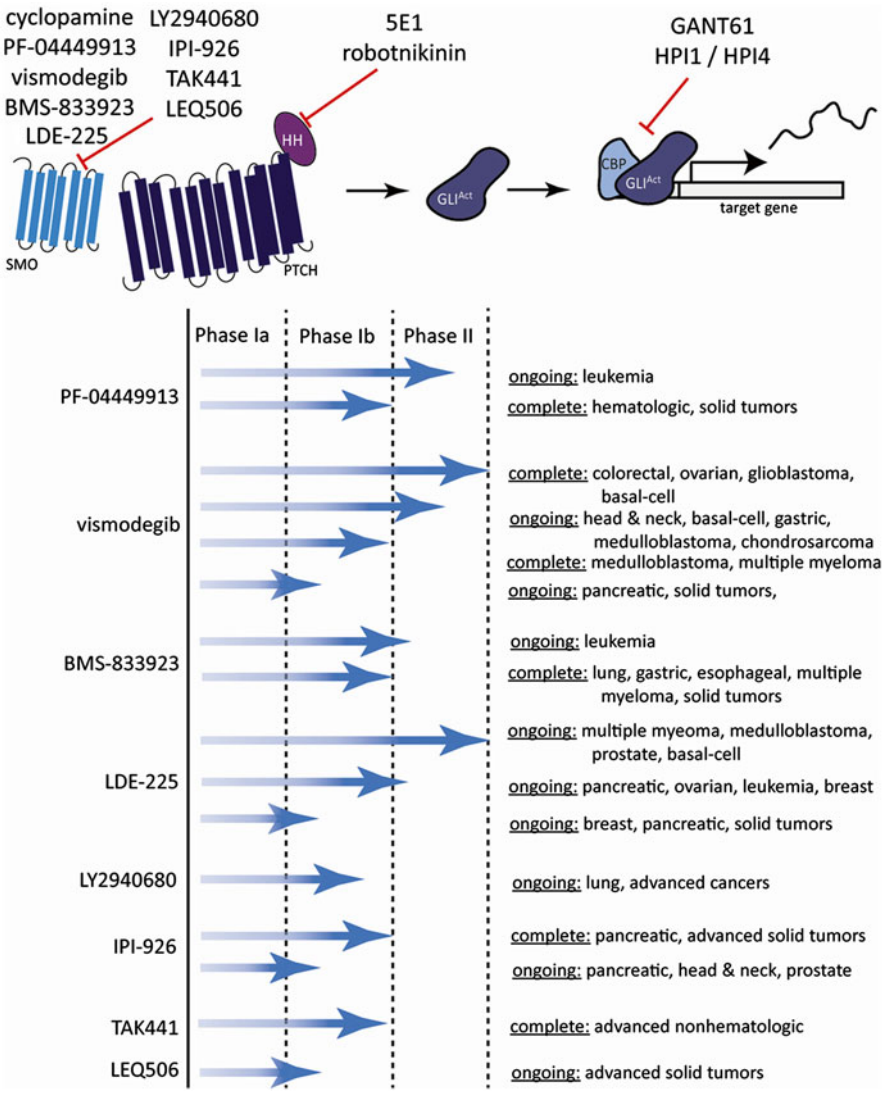

Fig. 15.6 Drug development against Hedgehog signaling focuses on Smoothened inhibition. The numerous anti-Hh agents in clinical testing almost exclusively target SMO. Vismodegib (anti-SMO) has already been approved by the FDA for the treatment of basal cell carcinoma. While resistant variants have been described, it appears that vismodegib resistance does not confer resistance to all SMO inhibitors. Pre-clinical testing of downstream targets suggests that the next line of anti-Hh therapeutics will have different modes of action than those already in clinical use.

TAK-441

Takeda Pharmaceutical Company, Ltd. modified a previous molecule to generate TAK-441 with an improved pharmacological profile including increased potency and bioavailability (Ohashi et al. 2012a, b). TAK-441 binds to SMO and blocks Hh signal transduction (Ishii et al. 2013). Preclinical profiling revealed anti-tumor effects in a murine model of medulloblastoma and in castration-resistant prostate xenografts (Ohashi et al. 2012a; Ibuki et al. 2013). It may be possible to use GLI1 mRNA expression (a target of Hh transcriptional response) as a biomarker to predict the effect of TAK-441 in clinical trials (Fig. 15.6) (Kogame et al. 2013).

LEQ506

Novartis has led the development of a SMO inhibitor, LEQ506 (Fig. 15.6). When compared to sonidegib (LDE225, another Novartis-lead pharmaceutical), LEQ506 has improved aqueous solubility, increased potency against a mouse model of medulloblastoma, and increased inhibition of GLI-dependent transcription. LEQ506 was effective against a SMO-mutant and vismodegib-resistant cell line. LEQ506, however, has a shorter half-life than sonidegib and requires a higher dosage (Peukert et al. 2013).

LY 2940680 (Taladegib)

Taladegib inhibits the Hh pathway by directly binding to Smo (Wang et al. 2013; Bai et al. 2014). This was observed in human xenograft and murine models of medulloblastoma. It was effective against the D473H-mutant cell line which is resistant to vismodegib (Bender et al. 2011).

SANT1-4

A small-molecule compound screen identified four molecules (SANT1-4) which modulate SMO activity. SANT1 and SANT2 have been demonstrated to lock SMO into an inactive state, preventing its engagement of downstream Hh signaling (Rohatgi et al. 2009).

IPI-926

Developed by Infinity Pharmacetuticals Inc., IPI-926 (saridegib, Fig. 15.6) is a semi-synthetic analogue of cyclopamine. Preclinical profiling revealed improved potency and pharmacokinetic profile relative to cyclopamine. IPI926 induced complete tumor regression in a Hh-dependent medulloblastoma allograft model (Tremblay

et al. 2009). Treatment prolonged overall survival in a similar model and was active against the D473H point mutation (Lee et al. 2012). In phase I study, IPI-926 was well tolerated and a response was observed in one third of patients (Jimeno et al. 2013b).

LDE225

In phase I testing, LDE225 (sonidegib or erismodegib, Fig. 15.6) exhibited activity in advanced basal-cell carcinoma and relapsed medulloblastoma. Side effects were relatively mild, with the exception of elevated serum creatine kinase in 18 % of patients. Reduction of GLI1 mRNA was observed in a dose-dependent manner (Rodon et al. 2014). Further clinical testing will identify if the effects of LDE225 can be translated to other cancer types.

5.2.3 GLI-Mediated Transcription Inhibitors

GANT58 and GANT61

GANT (GLI ANTagonist)-58 and GANT61 were identified in a small-molecule screen described by Lauth et al. (2007). GANT58 has a thiophene core with four pyridine rings. Inhibition of GLI-mediated transcription by GANT58 in acute T-cell leukemia showed anti-cancer activity and demonstrated reduced viability of T-ALL cells (Hou et al. 2014). Treatment of prostate cancer xenografts with GANT58 contributed to the development of stable disease in mice; however, GANT61 was more potent in initial testing. The vast majority of pre-clinical studies have thus focused on GANT61. GANT61 is a hexahydropyrimidine derivative shown to inhibit Hh signaling and reduce tumor growth of prostate cancer cells (Lauth et al. 2007). It is suggested that GANT61 alters the conformation of GLI1 and as a result compromises DNA binding of GLI1 (Lauth et al. 2007). Treatment with GANT61 has been effective against Eweing Sarcoma cells, biliary tract carcinoma, lung squamous carcinoma, and PDAC (Xu et al. 2013; Huang et al. 2014; Matsumoto et al. 2014).

HPI1 and HPI4

Four HPI (Hedgehog Pathway Inhibitor) molecules were identified in a small-molecule screen conducted by Hyman et al. They describe two of these compounds, HPI1 and HPI4, as modulators of GLI-dependent transcription. Both HPI1 and HPI4 affect the stability and processing of GLI1 and GLI2 (Hyman et al. 2009). Most recently, HPI1 has been packaged in a polymeric nanoparticle (NanoHHI) and shown to inhibit the growth of pancreatic and hepatocellular carcinoma xenografts (Chenna et al. 2011). NanoHHI treatment inhibited the expression of CD133, which marks a subpopulation of hepatocellular carcinoma CSCs (Xu et al. 2011).

5.2.4 Conclusions

Most of the side-effects of anti-Hh therapy have been mild (Amakye et al. 2013). The agents which have progressed into clinical testing almost exclusively target SMO. While several of them are effective against cancers which are resistant to first-line SMO-inhibitor vismodegib, further resistance will require agents which target other aspects of the pathway.

6 Cross-Talk Between Signaling Pathways

The development of an entire organism through several signaling pathways requires extensive cooperation, or cross-talk, between them. These interactions represent additional layers of complexity in targeting stem cell signaling in cancer, as inhibition of signaling through one pathway may lead to compensation via the remaining pathways.

Crosstalk between stemness pathways has been described and can occur by several mechanisms (Guo and Wang 2008; Javelaud et al. 2012). First, there may be physical interactions between components of two pathways (e.g. Wnt effector, DVL inhibits Notch) (Axelrod et al. 1996). The GLI3 repressor protein can interact with β-catenin and prevent transactivation (Fig. 15.7) (Ulloa et al. 2007).

Next, one component may be an enzymatic or transcriptional target of another pathway. Both Hh and Wnt signaling result in transcription of genes which are Notch-receptor ligands. One transcriptional target of Hh signaling is JAG2, while a target of TCF/LEF transcription is JAG1 (Fig. 15.7) (He et al. 2006). Wnt signaling also results in the transcription of the Hh repressor protein, GLI3 (Alvarez-Medina et al. 2007). Alternatively, GLI proteins allow Hh to induce Wnt signaling as the WNT proteins are targets of GLI-mediated transcription (Mullor et al. 2001; Yang et al. 2009). This Hh-induced Wnt signaling has been observed in pancreatic cancer models (Pasca di Magliano et al. 2007).

Finally, one pathway may compete with or modulate a mediator of the other pathway. For example, SUFU can inhibit both activating GLI proteins (Hh signaling) and β-catenin (Wnt signaling). Hh signaling has been reported to up-regulate a Wnt antagonist, secreted frizzled-related protein 1 (SFRP1), resulting in inhibition of Wnt signaling (Fig. 15.7) (He et al. 2006).

A number of publications have identified additive growth suppression when more than one stem-cell pathway is inhibited. For example, simultaneous inhibition of Hh and Notch in leukemia, pancreatic and prostate cancer suggests these pathways cooperate in cancer progression as additive suppressive effects are observed (Ristorcelli and Lombardo 2010; Okuhashi et al. 2011). Similarly, inhibition of the TGF-β and Notch pathway suggests that these pathways cooperate in EMT (Guo and Wang 2008).

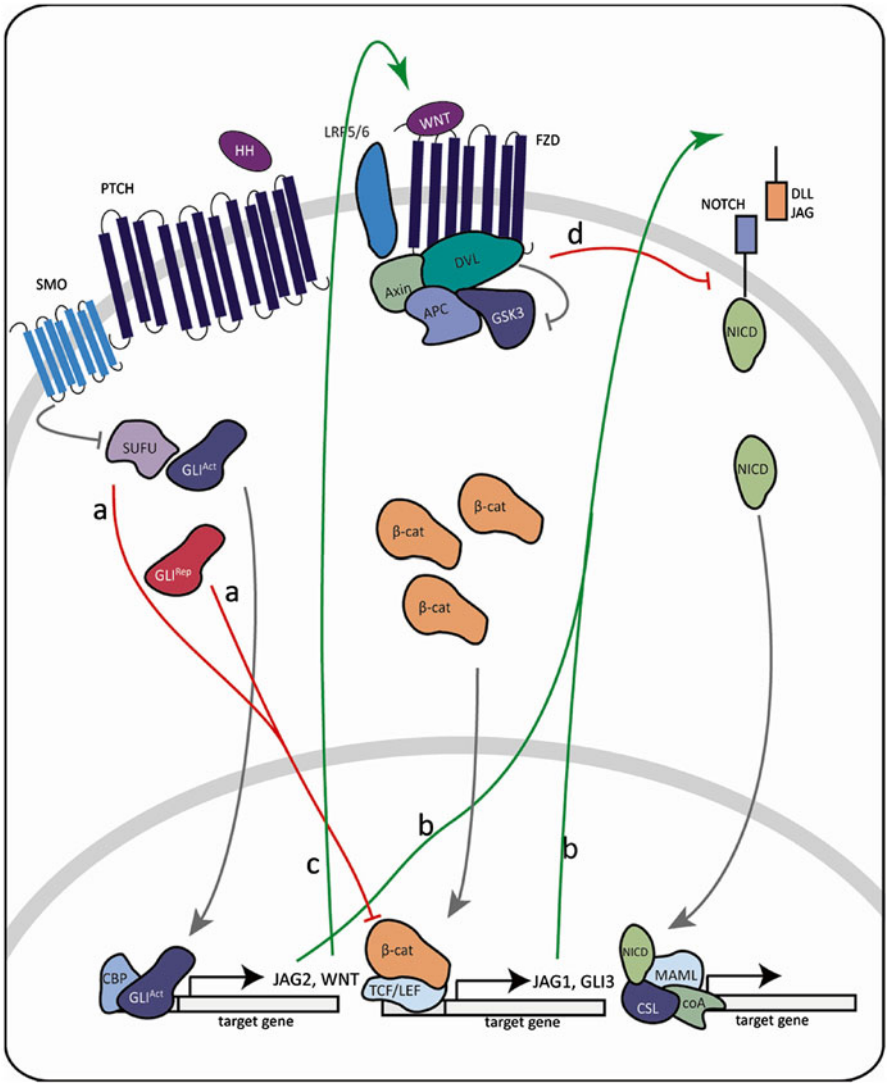

Fig. 15.7 Stemness pathways exhibit numerous points of "cross-talk". A few interactions between the Notch, Wnt, and Hh pathways are depicted here. **(a)** First, both Sufu and repressive GLI proteins (Hh signaling) can inhibit the activation of transcription by β-catenin (Wnt signaling). **(b)** Next, transcriptional targets of Hh and Wnt signaling act as ligands for the Notch receptors. **(c)** Wnt ligands are also transcriptional targets of Hh signaling, suggesting that Hh can activate Wnt signaling. **(d)** Finally, Dishevelled (DVL) can inhibit the function of NICD. These interactions demonstrate that stem cell signaling is a convoluted network of multiple pathways.

7 Molecules with Pan-inhibitory Effects

7.1 Genistein

Genistein (4,5,7-trihydroxyisoflavone) is an isoflavone phytoestrogen, derived from *Genista tinctoria*. A variety of evidence indicates that genistein can inhibit Notch signaling (Wang et al. 2005; Pan et al. 2012; Dandawate et al. 2013). The precise mechanism is unknown; however, it may be due to miR-34a up-regulation (Xia et al. 2012a). In phase I testing, isoflavone supplementation in prostate cancer patients revealed no toxicity (Miltyk et al. 2003; Takimoto et al. 2003; Fischer et al. 2004). An analog of genistein, phenoxodiol, inhibited breast cancer development in a rat model (Constantinou et al. 2003). Interestingly, it has also been demonstrated to enhance the activity of conventional chemotherapy drugs (Alvero et al. 2006). Further efficacy testing is necessary before any conclusions can be made about the use of genistein or its derivatives in human cancers.

7.2 Curcumin

Curcumin is a diarylheptanoid and a natural phenol. It is the principle curcuminoid of turmeric. It has poor bioavailability as it is insoluble in water. Inhibition of Wnt signaling has been described in osteosarcoma, liver, breast, and colon cancers, resulting in potent growth inhibition (Jaiswal et al. 2002; Prasad et al. 2009; Leow et al. 2009; Kim et al. 2013a). Natural analogs of curcumin down-regulated p300, an essential positive regulator of Wnt signaling (Ryu et al. 2008). Intriguingly, activation of Wnt by curcumin has also been described in neuroblastoma cells and in adipocytes (Ahn et al. 2010; Zhang et al. 2011a), suggesting that further characterization is required to determine in which contexts curcumin can be used to inhibit Wnt signaling. Evidence suggests that curcumin may also modulate Notch signaling by down-regulating Notch1 (Subramaniam et al. 2012; Li et al. 2012). The growth-inhibitory effects observed may be due to crosstalk with the NFκβ pathway (Wang et al. 2006). The preventative effects of curcumin have also been investigated in a phase IIa trial of patients at high risk for developing colorectal cancers. Patients receiving curcumin had a lower number of aberrant crypt foci, suggesting that high-risk patients may benefit from curcumin as a preventative treatment (Carroll et al. 2011). Curcumin has also been observed to inhibit Hh signaling (Elamin et al. 2009; Slusarz et al. 2010; Sun et al. 2013). These pan-inhibitory effects of curcumin make it a particularly appealing natural molecule for cancer therapy. Modifications to the structure of curcumin may increase its bioavailability and potency, thus enhancing its anti-cancer effects.

7.3 Resveratrol

Resveratrol (*trans*-3,5,4′-trihydroxystilbene) is a natural phenol and a member of the phyoalexin family. It is found in red grapes, wine, nuts, and several plants. A number of its anti-cancer effects have been attributed to inhibition of topoisomerase activity, or its estrogen-antagonizing structure (Bowers et al. 2000; Leone et al. 2012; Basso et al. 2013).

Interestingly, several studies have described activation of Notch signaling by resveratrol in carcinoid, medullary thyroid cancer, and glioblastoma cells, inducing apoptosis (Pinchot et al. 2010; Truong et al. 2010; Lin et al. 2011). A separate study, however, observed resveratrol-mediated inhibition of Notch signaling in T-ALL, which induced apoptosis (Cecchinato et al. 2007). Similar effects were seen in cervical cancer cells; however, selective Notch inhibition did not achieve the same result (Zhang et al. 2014). The authors suggest that concurrent inhibition of Notch, Wnt, and STAT3 signaling resulted in the observed apoptotic effects of resveratrol. Additional studies have demonstrated obstruction of Wnt signaling by resveratrol (Hope et al. 2008; Vanamala et al. 2010). Many of these have focused on colon cancer, likely due to the importance of APC and Wnt signaling in FAP. A 2012 study determined that resveratrol inhibits the formation of the β-catenin/TCF complex, thus modulating transcription initiation at target genes (Chen et al. 2012a). Phase I trials of resveratrol have demonstrated inhibition of Wnt signaling in normal colonic mucosa; and, using a micronized formulation, increased apoptosis of hepatic metastases (Nguyen et al. 2009; Howells et al. 2011). In human trials, the major dose-limiting side effect of resveratrol has been gastrointestinal toxicity (la Porte et al. 2010; Brown et al. 2010). Resveratrol may also inhibit Hh signaling. While the mechanisms range from decreased nuclear translocation of GLI and decreased transcription of target genes to down-regulation of PTCH and SMO, resveratrol has been described to modulate Hh signaling in AML, prostate cancer, and pancreatic cancer (Slusarz et al. 2010; Su et al. 2013; Qin et al. 2014).

A major limiting factor in the clinical use of resveratrol is its poor bioavailability (Walle 2011). While resveratrol is easily absorbed, it is extensively metabolized in the intestine and liver resulting in limited efficacy. The use of methylated derivatives of resveratrol may decrease clearance of resveratrol by increasing metabolic stability and result in improved anti-cancer effects of resveratrol (Walle et al. 2007; Cai et al. 2010).

7.4 Celastrol

Celastrol (tripterene) is a triterpenoid, isolated from the root extracts of *Tripterygium wilfordii* (Thunder god vine) and *Celastrus regelii*. It has been described to have anti-oxidant, anti-inflammatory, and anti-cancer activity (Allison et al. 2001). Some

of its anti-cancer effects may be a result of its modulation of Notch signaling, as treatment of leukemia cells resulted in a down-regulation of Notch1 (Wang et al. 2010). Interestingly, celastrol has been described to induce apoptosis via the activation of Wnt signaling. In colorectal cancer cells, celastrol increased nuclear beta-catenin levels (Lu et al. 2012).

7.5 Honokiol

Honokiol is a small-molecule polyphenol, isolated from various components of trees belonging to the genus *Magnolia*. It has been shown to have anti-inflammatory, anti-angiogenic, and anti-cancer properties (Fried and Arbiser 2009). Treatment with honokiol in preclinical models can modulate Wnt signaling, and may have CSC-specific effects. In oral squamous cell carcinoma CSCs, honokiol decreased β-catenin and a down-regulation of downstream targets was observed (Yao et al. 2013). Similar effects were seen in non-small cell lung cancer cells. Antagonism of the Notch pathway has also been observed following honokiol treatment. In a colon cancer model, honokiol sensitized CSCs to ionizing radiation. The expression of components of the γ-secretase complex as well as downstream target genes were reduced (Ponnurangam et al. 2012). The effects of honokiol could be reversed by the addition of NICD, suggesting that Notch signaling is vital for this response. A similar decrease in γ-secretase components was observed when melanoma cells were treated with honokiol (Kaushik et al. 2012).

7.6 Arsenic Trioxide

Arsenic has been used as a medicinal agent for thousands of years. Currently, arsenic trioxide (ATO) is used in combination with all-trans retinoic acid in the treatment of acute promyelocytic leukemia (APL). ATO promotes cellular differentiation, induces apoptosis in malignant and normal cells, and induces an accumulation of reactive oxygen species (Rojewski et al. 2002; List et al. 2003; Park et al. 2005). These effects may be mediated by inhibition of the Notch pathway. In gliomas, treatment with ATO resulted in decreased transcription of Notch-dependent genes. This was accompanied by a depletion of the CSC population (Zhen et al. 2009). Similar results have been observed in breast cancer and glioblastoma (Xia et al. 2012b; Wu et al. 2013). ATO may also antagonize Hh signaling (Raju 2010; Kim et al. 2013b). In a mouse model of Hh-dependent medulloblastoma, ATO treatment improved survival (Beauchamp et al. 2010). It is suggested that ATO binds GLI1 and inhibits its transcriptional activity; however, a separate study observed an ATO-induced reduction of GLI2 (Kim et al. 2010a). It is likely that the effects of ATO on the Hh pathway are mediated by the GLI proteins, and further experimentation will elucidate the precise mechanisms.

8 Conclusion and Future Perspectives

8.1 Roadblocks to Success

8.1.1 Preclinical/Clinical Failures

Drug development for stemness pathways closely follows that for many other targets. The vast majority of therapeutic agents remain in preclinical studies, and a number of agents which show promise in preclinical models fail in clinical trials. These disappointments may be due to any number of differences between preclinical and clinical testing. Cell line models lack the inherent heterogeneity of human cancers, and the use of xenograft models requires immunocompromised hosts. Neither of these popular preclinical paradigms properly recapitulates the complexity of treating patients.

It will be important to require the same success in preclinical models as we require in clinical settings – if clinical success is defined as inducing tumor regression or stable disease, then slowing tumor growth in preclinical tests is insufficient. The interesting concept of co-clinical trials presents an opportunity to hasten the progress of targeted therapies (Nardella et al. 2011; Chen et al. 2012b). In principle, co-clinical trials encompass a genetically-engineered murine model paralleling a human clinical trial. This allows real-time feedback on treatment failures and successes, and simultaneous integration of preclinical and clinical data.

8.1.2 Strategies to Overcome Resistance

This approach to clinical testing of targeted therapies will allow rapid redeployment of alternate therapies when resistance develops. While targeting stemness pathways is a relatively young field of anti-cancer therapy, it is not surprising that resistance to a number of these therapeutic agents has already been described. Indeed, it is most surprising that the emergence of resistance has not altered the strategies being used to target stemness pathways. The success of imatinib (Novartis) in treating BCR-ABL CML was followed quickly by the emergence of resistant variants (Valent 2007). This necessitated the development of second-generation tyrosine kinase inhibitors (dasatinib, nilotinib, and bosutinib) and third-generation ponatinib (Golas et al. 2003; Lombardo et al. 2004; Weisberg et al. 2005). Finally, a novel treatment for CML (omacetaxine), which acts independently of BCR-ABL tyrosine kinase inhibition, was developed; it has shown promise in treating patients who have failed first- and second-generation tyrosine-kinase-inhibitor therapy and was approved by the FDA in 2012 (Pérez-Galán et al. 2007).

The development of anti-SMO therapies to inhibit Hh signaling mimics the BCR-ABL story. Mutations have already been described which confer resistance to the first-line vismodegib (Metcalfe and de Sauvage 2011; Chang and Oro 2012), and while other SMO-antagonists may still be effective, it is likely only a matter of

time before resistance to second- and third-line antagonists emerges. It will be essential to hurry the development of therapies which target other aspects of the Hh signaling pathway as the SMO-antagonists move into wider clinical use (Metcalfe and de Sauvage 2011). In the Hh pathway, it may be essential to use a therapeutic such as GANT61 to target GLI-mediated transcription once resistance emerges at the SMO-level (Fig. 15.6) (Matsumoto et al. 2014). Therapeutic agents which target different aspects of the Notch, Wnt, and Hh pathways are in various stages of development – while some classes of drugs, such as the Notch-targeted GSIs or the Hh-targeted SMO antagonists, are further ahead, the emergence of resistance will place a selective pressure on those less-developed agents. Alternatively, resistance to these targeted therapies may be addressed by combining anti-stemness agents with other specific agents. In SMO-antagonist-resistant tumors, this may mean the addition of a PI3K-inhibitor (Kim et al. 2010b, 2013b).

8.1.3 Dealing with On-Target Side Effects

It is important to recognize that even targeted therapies have serious on- and off-target side effects. For example, a number of CML patients treated with imatinib developed congestive heart failure (Kerkelä et al. 2006). This was caused by a build-up of misfolded proteins in the endoplasmic reticulum, activating apoptosis. Inhibiting the BCR-ABL fusion protein also systemically inhibits the function of the ABL tyrosine kinase, leading to imatinib's particular effects on cardiac function.

The clinical use of GSIs for leukemia patients and those with solid tumors exposed the importance of considering on-target side effects of anti-stemness agents. While the Notch, Wnt, and Hh pathways are vital for embryonic patterning and development, they are also active in many adult stem cell populations. Treatment with GSIs led to gastrointestinal toxicity due to the involvement of Notch in the intestinal tract (Searfoss et al. 2003; Milano et al. 2004; Wei et al. 2010). The resulting dose-limiting goblet cell hyperplasia has curtailed the use of GSIs in clinical settings and modified dosing schedules have been investigated (Krop et al. 2012). The use of steroidal agents in combination with GSIs has also been investigated and seems promising (Real et al. 2008).

Similarly, on-target side effects have been observed in patients treated with Wnt signaling antagonists. Wnt and Hh signaling cooperate extensively in regulating bone turnover; thus, the use of targeted therapies in these pathways has resulted in abnormal bone mass. The FDA halted clinical testing of two anti-Wnt agents (ipafricept and vantictumab) until the on-target bone side effects were addressed. The use of zoledronic acid in these patients appears to mediate these effects.

Targeting stemness pathways will not be without consequence until a tumor-specific delivery platform can be mobilized. Ado-trastuzumab emtansine (Kadcycla or T-DM1, Genentech) consists of the Her2 monoclonal antibody, Herceptin, conjugated to a cyctotoxic agent, mertansine (Verma et al. 2012). Approved by the

FDA in 2013 for the treatment of metastatic Her2-positive breast cancer, T-DM1 exhibited a better safety profile and improved efficacy over trastuzumab alone. This is an important harbinger of the potential for tumor-specific delivery.

8.2 Evidence for Success

8.2.1 Immediate Clinical Successes

Several agents discussed in this chapter have already demonstrated clinical success, leading to FDA approval. Vismodegib (a SMO antagonist, Fig. 15.6) is approved for the treatment of locally-advanced or metastatic basal cell carcinoma, and demicizumab (anti-DLL4 agent, Fig. 15.2) received an orphan-drug designation for the treatment of pancreatic cancer. This demonstrates that targeted anti-stemness therapy is an active and successful field of drug development. Other agents in advanced stages of clinical testing, such as the Hh antagonist LDE225, the PF-03084014 GSI, and PRI-724, a CBP inhibitor, demonstrate benefits to patient outcomes.

Just as relevant, however, are those agents which have exhibited little-to-no clinical success. RO4929097, a GSI, has little benefit as a monotherapy, though it may still yet exhibit synergistic effects with conventional chemotherapies or even other targeted anti-Notch agents (Strosberg et al. 2012; De Jesus-Acosta et al. 2014). While some may call this a failure of the drug-development pipeline, it is important to consider how the success of anti-stemness agents is measured.

8.2.2 Measuring Long-Term Effects

It is difficult to evaluate the long-term efficacy of the targeted anti-Notch, Wnt, or Hh therapeutics discussed in this chapter, as many of them are fairly recent developments. An additional factor confounding the assessment of these agents is the rarity of the cell populations they target. CSCs often exhibit high signaling via these pathways when compared to the non-CSC component of the tumor. Importantly, however, the frequency of CSCs in many cancers is less than 1 %. Thus, targeting stemness pathways in human cancers may show little immediate success over conventional chemotherapy, as increased toxicity to 1 % of cells in a tumor is difficult to quantify. From another perspective, however, the hypothesized role for CSCs in cancer recurrence suggests that targeting CSCs may reduce recurrence rate and increase overall survival (Beck and Blanpain 2013).

Clinical testing of these targeted agents should include long-term follow-up as well as a determination of CSC frequency before, during, and after treatment. This data will allow us to determine if the overall efficacy of the agent can be attributed to anti-CSC effects.

8.3 The Future of Targeting Stemness Pathways

With the hypothesized importance of CSCs in tumorigenesis, metastasis, chemotherapy resistance, and recurrence gaining increasing credence (Bonnet and Dick 1997; Singh et al. 2004; Ginestier et al. 2007), there has been a major thrust to identify novel therapies that target CSCs. The intrinsic linkage of stem cell signaling pathways with CSC maintenance and tumorigenicity provides an avenue for therapeutic development and a more thorough study of CSCs in human cancers. The number of pre-clinical investigations and clinical trials examining the potential use of anti-stemness drugs has grown exponentially in recent years. The success of future trials will likely depend on extensive consideration of the cross-talk between stemness pathways. Future therapies may include dual-purpose agents such as the recently-described NL-103, a Hh and HDAC inhibitor (Zhao et al. 2014). Additionally, it is becoming increasingly apparent that the end result of signaling through these stemness pathways depends heavily on the cellular context – signaling may be oncogenic or tumor-suppressive. Even the use of a single agent can activate or inhibit signaling (e.g. resveratrol). The identification of patients who may benefit from these therapies or combinations of anti-stemness therapeutics will necessitate an evaluation of stemness pathway cross-talk in patient tumors. Additionally, altered clinical paradigms should be considered, such as co-clinical trials and outcome measures that incorporate CSC frequency measurements.

Targeted therapies have outpaced natural product research in terms of resources spent by pharmaceutical companies on the development of novel anti-stemness pathway drugs for cancer. We will learn in the coming years if this strategy was effective, or if a new shift in research focus may occur. It has been suggested that natural molecules with novel mechanisms are more likely to be successful than many small molecules targeted at the same interaction (Ganesan 2008). Major advances may come from identifying the targets of natural molecules with proven anti-stemness/cancer activity and utilizing this information to generate semi-synthetic natural compounds with enhanced activity or developing novel strategies for targeted therapy (Pucheault 2007).

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Chapter 16
Targeting Cancer Stem Cells and the Tumor Microenvironment

Alice Turdo, Matilde Todaro, and Giorgio Stassi

Abstract Compelling evidence indicates that the survival and behavior of cancer stem cells (CSCs) are positively regulated by specific stimuli received from the tumor microenvironment, which dictates the maintenance of stemness, invasiveness, and protection against drug-induced apoptotic signals. CSCs are per se endowed with multiple treatment resistance capabilities, thus the eradication of CSC pools offers a precious strategy in achieving a long-term cancer remission. Numerous therapies, aimed at eradicating CSCs, have been elaborated such as: (i) selective targeting of CSCs, (ii) modulating their stemness and (iii) influencing the microenvironment. In this context, markers commonly exploited to isolate and identify CSCs are optimal targets for monoclonal antibody-based drugs. Furthermore, the molecules that inhibit detoxifying enzymes and drug-efflux pumps, are able to selectively suppress CSCs. Auspicious outcomes have also been reported either by targeting pathways selectively operating in CSCs (e.g. Hedgehog, Wnt, Notch and FAK) or by using specific CSC cytotoxic agents. Other compounds are able to attenuate the unique stemness properties of CSCs by forcing cell differentiation, and this being the case in ATRA, HDACi, BMPs and Cyclopamine, among others. Targeting the interplay between paracrine signals arising in the tumor stroma and the nearby cancerous cells via the inhibition of VEGF, HIF, CD44v and CXCR4, is increasingly recognized as a significant factor in cancer treatment response and holds allurring prospects for a successful elimination of CSCs. In the present chapter, we discuss the latest findings in the optimization and tailoring of novel strategies that target both CSCs and tumor bulk for the eradication of malignancies.

Keywords Cancer stem cells • Tumor microenvironment • Cancer therapy • Stemness modulator drugs • Targeted therapy

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1 Introduction

The concept that tumors are hierarchically organized and harbor cells with distinct tumor-initiating capabilities and self-renewal potential, referred to as cancer stem cells (CSCs), has long been observed in a variety of hematopoietic malignancies and solid tumors and is now well-recognized by the scientific community (Valent et al. 2012). By virtue of their innate plasticity, it is worth considering that CSCs fuel and succeed in tumor growth, treatment resistance, distant metastasis formation and patient relapse. Mechanistically, CSCs share several biological properties with normal adult stem cells that endow them with a survival advantage upon chemotherapeutic intervention. These include dormancy (quiescence), active DNA repair machinery, an enhanced reactive oxygen species (ROS) defence capability, a higher expression of multiple drug resistance (MDR) membrane transporters and anti-apoptotic proteins (Maugeri-Sacca et al. 2011; Zhou et al. 2014).

Thus, attractive emerging strategies have been developed to selectively target CSCs by using agents directed at CSC-surface markers, drug-detoxifying enzymes, drug efflux pumps or key signaling pathways sustaining the stemness properties of CSCs. Otherwise, stemness modulator drugs force CSCs to differentiate terminally, resulting in the loss of self-renewal potential and the gaining of susceptibility to cytotoxic therapies. To eventually overcome cancer resistance and relapse, a simultaneous delivery of stem cells targeting drugs or stemness modulator compounds, has been tested in combination with standard anticancer drugs to successfully eliminate CSCs, tumor bulk cells and spontaneously dedifferentiated non-CSCs (Chen et al. 2012; Chaffer et al. 2011). Of note, stem cell targeting drugs eradicate CSCs but at concentrations less toxic to non-CSCs. Conversely, stemness inhibiting drugs aim at reducing the stemness of CSCs and uniquely, at high doses, they may eliminate CSCs and non-CSCs with similar potency. Finally, paracrine signals between cancer cells and stromal cells are required to trigger an epithelial-to-mesenchymal transition (EMT) program. Besides the acquisition of a mesenchymal and invasive state, EMT seems to confer stem-like properties to neoplastic epithelial cells (Morel et al. 2008), and subsequently additional autocrine signals, arising from cancerous cells themselves, appear to maintain this mesenchymal state (Scheel et al. 2011). Therefore, specific molecular therapies that target CSC peculiarities and prominent tumor microenvironment signals may be powerful determinants in tumor shrinkage and successful elimination of CSCs (Fig. 16.1).

2 Selective Cancer Stem Cells Targeting Drugs

Proof of evidence that CSCs are endowed with self-renewal and differentiation capabilities is represented by the ability to engraft tumors when serially transplanted in immunocompromised mice. Further support, recently emerging from in vivo genetic cell fate tracking experiments, confirmed the capability of CSCs to seed a

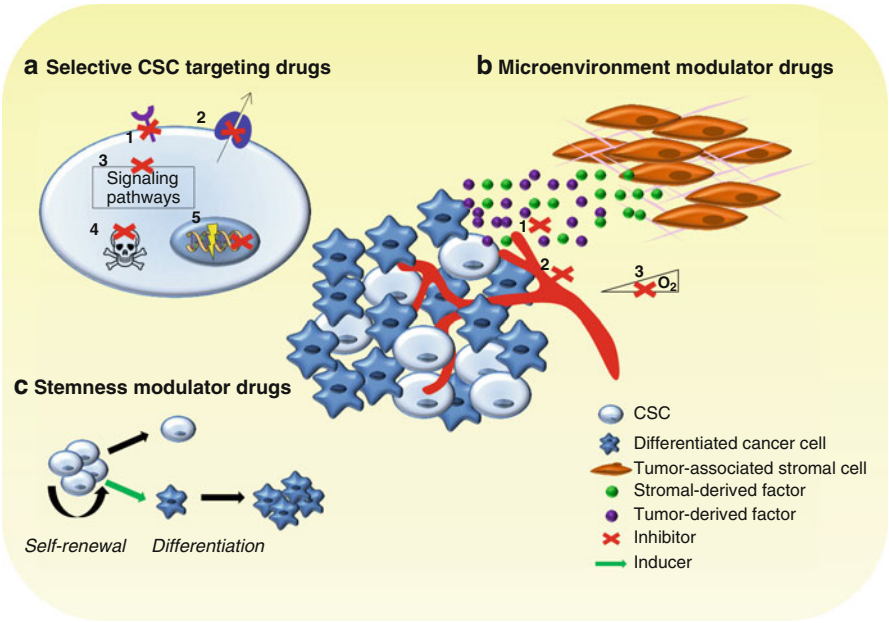

Fig. 16.1 *Targeting cancer stem cells and the tumor microenvironment*. (**a**) Therapeutic approaches to selectively target CSCs use mAbs directed to CSC-surface markers (1), agents blocking drug efflux pumps (2), inhibitors of signaling pathways that take part in controlling the fate of CSCs (3), CSC-specific cytotoxic compounds (4) and inhibitors of the DNA repair machinery (5). (**b**) Microenvironment modulator drugs can impair the effect of stromal- and cancer-derived factors (1), inhibit angiogenesis (2) and counteract the pro-oxidant environment generated by tumor hypoxia (3). (**c**) Stemness modulator compounds force the differentiation of CSCs and in combination with standard chemotherapy contribute to the successful elimination of CSCs and tumor bulk. CSC: Cancer stem cell, mAB: monoclonal antibody

tumor and recapitulate its heterogeneity (Zhu et al. 2014; Schepers et al. 2012). The criteria used to identify CSCs in solid tumors and hematopoietic disorders include certain in vitro properties among which (i) CSCs can be distinguished and isolated with specific cell-surface marker profiles or intracellular molecules, (ii) CSCs are endowed with increased resistance to chemotherapeutic compound (CSCs are detectable for their high levels of detoxify enzymes and MDR) and (iii) the activation of CSCs-dependent pathways, which could offer a functional marker for their identification (Pattabiraman and Weinberg 2014).

2.1 CSC Surface Markers As a Therapeutic Target

Thus, the ability to use CSCs' peculiar surface markers has been suggested as a promising therapeutic approach. One must bear in mind that some limitations do exist such as, the existence of inter- intra- tumor heterogeneity and splicing variants,

the different methodologies used for CSCs detection and the presence of some common markers shared by normal adult stem cells. For instance, CD44 is a transmembrane glycoprotein and the receptor for hyaluronic acid (HA) and osteopontin (OPN), among others. It is expressed in CSCs from distinct solid tumor types and H90, an anti-CD44 monoclonal antibody (mAb), was the first antibody that showed CSC targeting properties. In vivo administration of H90 interfered with acute myeloid leukemia (AML) stem cells' homing capability in the microenvironmental niche and maintained their stem cell status (Jin et al. 2006). Similarly, in a xenograft model initiated by triple negative breast cancer cells, the anti-CD44 mAb P245 inhibited tumor growth and recurrence if injected during the apparent tumor remission period achieved after treatment with doxorubicin and cyclophosphamide (Marangoni et al. 2009).

GV5 is a recombinant human mAb that recognizes the extracellular domain of CD44's alternative splicing variant, termed CD44R1 (v8-v10). In athymic mice GV5 inhibited tumor formation, after the subcutaneous transplantation of larynx and cervix cancer cells, due to the induction of antibody-dependent cellular cytotoxicity (ADCC) and internalization of CD44R1 (Masuko et al. 2012). H4C4 is an anti-CD44 mouse mAb that decreased pancreatic CSC capabilities of in vitro tumor sphere formation and in vivo tumor growth. It also impaired metastasis formation and recurrence after radiotherapy via Nanog and STAT3 signaling pathway inhibition (Li et al. 2014). Finally, due to its promising preclinical results, RO5429083, which is a humanized mAb directed against an extracellular epitope of human CD44, has been evaluated in a phase I clinical study on CD44-expressing metastatic and/or locally advanced solid tumors. Another phase I clinical study is still ongoing involving patients with AML (http://www.cancer.gov/clinicaltrials).

MT110 is a bispecific bifunctional T-cell-engaging (BiTE) antibody that concomitantly binds to the epithelial cell adhesion molecule (EpCAM), a common CSC marker, and to the T-cell receptor complex CD3 which, leads to the activation of cytotoxic T-cells against EpCAM-expressing cells and causes cell death via redirected lysis. MT110 reduced the capacity of colon and pancreatic CSCs, co-cultured with peripheral blood mononuclear cells (PBMCs) as source of T-cells, to form spheres in vitro and to generate tumors in vivo (Herrmann et al. 2010; Cioffi et al. 2012). MT110, is in early stages of clinical trials for patients with locally advanced, recurrent or metastatic solid tumors, known to widely express EpCAM (http://www.cancer.gov/clinicaltrials).

Catumaxomab is a bispecific trifunctional antibody (Triomabs) binding to EpCAM and the CD3 complex in T-cells. In addition, it binds macrophages, natural killer (NK) and dendritic cells via its Fc fragment thus, synergizing the anti-tumor effects exerted by T-cells. When Catumaxomab is administered to patients with advanced solid cancers and suffering from malignant ascites, it activated peritoneal T-cells, stimulated the release of proinflammatory Th1 cytokines, decreased the peritoneal level of VEGF and eliminated $CD133^{+}/EpCAM^{+}$ CSCs (Jager et al. 2012). Catumaxomab has been approved in Europe for clinical use in the treatment of malignant ascites and the results, from a prospective randomized phase II/III

clinical trial, have been reported by Heiss et al. (2010). The ubiquitous expressed transmembrane antigen CD47 can trigger inhibition of phagocytosis (the so-called 'don't eat me' signal) on SIRPα-expressing phagocytic cells. CD47 blocking via the mouse mAb B6H12.2 favors the phagocytosis of human AML stem cells through mouse and human macrophages. Interestingly, B6H12.2 spares normal hematopoietic stem cells because they express low levels of CD47 (Majeti et al. 2009). 7G3 is a mouse mAb and recognizes the human interleukin-3 (IL-3) receptor α chain (CD123), which is overexpressed on AML blasts and $CD34^+$ AML stem cells. 7G3 inhibits the engraftment and homing of AML stem cells in immunocompromised mice through ADCC (Jin et al. 2009).

CSL362, a humanized anti-CD123 mAb with an increased affinity for human CD16, induces massive NK-mediated ADCC in both AML blasts and $CD34^+CD38^-CD123^+$ AML stem cells (Busfield et al. 2014). CSL362 is currently in the beginning stages of clinical trials for patients with AML (http://www.cancer.gov/clinicaltrials). A more detailed list of CSC specific markers and their use as putative therapeutic targets has been reviewed recently (Medema 2013; Naujokat 2014).

2.2 Targeting ABC Transporters in CSCs

ATP-binding cassette (ABC) transporters have been used to identify CSCs because they are overexpressed on the membrane of both normal and cancer stem cells. ABC transporters enable the efflux of drugs and are responsible for MDR. Thus, CSCs are able to expel the Hoechst 33342 dye by adopting such machinery and thus creating a 'side population' (SP) which, can be isolated by fluorescence-activated cell sorting (FACS). ABCB1 (P-glycoprotein), ABCG2 and ABCC1 are the most extensively studied ABC transporters in stem cell biology. In order to avoid drug resistance, much effort has been devoted to the design of ABC transporter inhibitors which, selectively eliminate CSCs but spare normal stem cells. However, several ABCB1 inhibitors, such as verapamil, tariquidar, and quinidine, have shown little efficacy in clinical settings. The elimination of CSCs has not been successful perhaps due to: clinical studies that were not designed correctly, the choice of an incorrect ABC transporter as a target and other combinations of CSC targeting drugs would have been preferable (Dean et al. 2005). Some ABCG2 inhibitors showed high toxicity both in vitro and in vivo. Novel compounds are in preclinical studies such as the ABCG2 inhibitor YHO-13351 which, sensitized the human cervical carcinoma cell line to irinotecan and reduced the CSC population (Shishido et al. 2013). Xia et al. developed an image-based high-content screening system and identified 12 potent high drug efflux cancer cell inhibitors from 1280 screened compounds. These inhibitors sensitized lung cancer cells to chemotherapeutic drugs and possibly affected in vivo tumorigenic capabilities of the CSC compartment (Xia et al. 2010).

2.3 Molecules That Inhibit Pathways by Sustaining CSCs

CSCs are dependent on activated signaling pathways different from those sustaining the bulk population. Therefore, targeting the stemness determinants could effectively conduct to the most durable remission and prevent resistance to chemotherapy and radiotherapy. Being an important player in self-renewal and maintenance of CSCs (Chakrabarti et al. 2014), the Wnt signaling pathway has been targeted by both small-molecule and biologic inhibitors. The first class of compounds includes ICG-001 which, acts as an antagonist of CREB-binding protein (CBP)/β-catenin (Emami et al. 2004) and showed to selectively eliminate drug resistant leukemic stem cells (Takahashi-Yanaga and Kahn 2010). Moreover, the small LGK974 (Liu et al. 2013) and IWP2 (Chen et al. 2009) molecules target the porcupine enzyme which, is responsible for palmitoylation of Wnt ligands, a required step in activating their secretion. A LGK974-based phase I clinical trial on patients with solid tumors is still ongoing (http://www.cancer.gov/clinicaltrials). The second class of compounds includes, the humanized mAb OMP-18R5 that binds to the extracellular domain of multiple Frizzled (FZD) receptors and blocks the Wnt3A-induced downstream pathway. In preclinical settings, it reduces tumorigenic capabilities of human breast, pancreatic, colon and lung cancer cells, compared to standard chemotherapy (Gurney et al. 2012), and is currently in its early stages of clinical trial for patients with solid tumors (http://www.cancer.gov/clinicaltrials). The activation of the Hedgehog (Hh) pathway is mandatory for the maintenance of CSC properties in various human cancers. The molecules antagonist of smoothened (SMO), a G protein-coupled transmembrane serpentine receptor that usually acts as a signal transducer of the proximal Hh pathway, such as GDC-0449, inhibit cell growth and induce apoptosis of pancreatic CSCs (Singh et al. 2011). Interestingly, the antineoplastic compound mithramycin, showed properties that target $Sox2^+$ medulloblastoma stem cells and bear the aberrant Sonic hedgehog (Shh) pathway activation. Specific to this context, although $Sox2^+$ cancer cells were driven by Shh signaling, they were not affected by either the Shh-targeted therapy with GDC-0449 or antimitotic chemotherapy. This suggests the existence of heterogeneity even within the Shh medulloblastoma subgroup and that a combination of bulk targeting drugs and CSCs targeted therapy could lead to a more notable control of the disease (Vanner et al. 2014). GDC-0449 is in phase II of the clinical trial regarding the treatment of basal cell carcinoma (http://www.cancer.gov/clinicaltrials).

The Notch signaling pathway is a well-recognized positive regulator of CSCs fate (Pannuti et al. 2010; Espinoza et al. 2013). The best way to target Notch activation, is to inhibit the proteolytic cleavage of the Notch intracellular domain (NICD) via the γ-secretase complex. γ-secretase inhibitors (GSIs) reduce self-renewal and tumorigenicity of GSCs and breast CSCs (Fan et al. 2010; Kondratyev et al. 2012). A phase I/II clinical trial that foresees the use of GSIs MK-0762 followed by docetaxel, whose purpose is killing breast cancer stem cells in advanced or metastatic breast cancer, has recently been completed (Schott et al. 2013). Antibodies targeting the Notch ligand Delta-like 4 (Dll4) such as the humanized mAb

OMP-21M18, have been developed and efficiently reduced CSC frequency in solid tumors (Hoey et al. 2009; Fischer et al. 2011). A comprehensive analysis of all ongoing and completed Notch clinical trials has recently been published (Andersson and Lendahl 2014). FAK activity seems to be critical for survival, migration and resistance to chemotherapy of CSCs (Sulzmaier et al. 2014; Schober and Fuchs 2011). Kang et al. demonstrated that the FAK inhibitor VS-6063 (which inhibits FAK autophosphorylation) overcomes resistance to paclitaxel in ovarian cancer by decreasing the AKT-dependent YB-1 phopshorylation which, in turn down-regulates the CD44 expression (Kang et al. 2013). Others showed that the up-regulation of CD44 favors breast cancer cell self-renewal, tumorspheres formation and induces paclitaxel resistance (To et al. 2010). Furthermore, CD44 up-regulates Nanog, responsible for increased ABCB1 expression and ovarian cancer cells acquired resistance to paclitaxel (Bourguignon et al. 2008). VS-6063 is currently in phase II of its clinical trial for *K-RAS* mutant non small cell lung cancer (NSCLC) patients. Similarly, other FAK inhibitors such as VS-4718 and PF-00562271, are in phase I of clinical evaluation (http://www.cancer.gov/clinicaltrials). Finally, the BMI-1 inhibitor PTC-209, has recently been proposed as an interesting small molecule affecting self-renewal of colorectal cancer cells with no systemic toxicity in preclinical settings (Kreso et al. 2014).

2.4 Agents That Selectively Eradicate CSCs

A high-throughput screen for agents that selectively kill CSCs has been performed by Gupta et al. Among a library of 16,000 compounds tested, salinomycin induced breast CSC-specific toxicity. Breast cancer cells were initially forced to undergo an EMT by means of an E-cadherin knockdown. Pre-treatment with salinomycin inhibited tumorsphere formation in vitro and reduced tumor seeding ability in vivo by >100-fold, compared to paclitaxel. Salinomycin treatment also decreased tumor mass and metastasis and increased epithelial differentiation of breast CSCs in an immunocompromised mouse model (Gupta et al. 2009). Successively, similar results have been reached in some type of cancers, including leukemia, colorectal cancer, lung cancer, GIST and osteosarcoma. Some findings also suggested that, a combination of salinomycin and conventional cytotoxic drugs could be a much more efficient strategy than the use of a single agent to improve therapeutic outcomes (Bardsley et al. 2010; Koo et al. 2013). Moreover, being that salinomycin seems to be toxic to normal stem cells at concentrations also effective in CSCs (Boehmerle and Endres 2011) it will render its clinical use as a single agent difficult. Salinomycin acts as a K^+ ionophore in biological membrane that promotes mitochondrial and cytoplasmic K^+ efflux however, the exact mechanisms underlying its toxicity against CSCs still remains unclear. It has been shown that salinomycin is a powerful inhibitor of the multidrug resistance protein 1 (MDR-1) (P-glycoprotein/ABCB1) (Riccioni et al. 2010). It inhibits the phosphorylation of the Wnt co-receptor LRP6, induces apoptosis in chronic lymphocytic leukemia (Lu

et al. 2011) and is an antagonist of the mTORC1 signaling pathway in breast and prostate cancer cells (Lu and Li 2014). On the other hand, it encourages ROS production and inhibits oxidative phosphorylation in mitochondria (Ketola et al. 2012), resulting in the possible elimination of CSCs, which rely on this metabolic process. In addition, recent studies have unveiled that salinomycin induces cell growth inhibition and apoptosis in multi drug resistant ovarian cancer cell lines, by ablating the activity of the signal transducer and activator of transcription 3 (Stat3) and thus, diminishing the expression of Stat3 target genes, such as *cyclin D1, S-phase kinase-associated protein 2 (SKP2)* and *SURVIVIN* (Koo et al. 2013). This is not surprising if we consider the most recent evidence which highlights the major role that Stat3 plays in reducing the effectiveness of drugs treatment. Specifically, the inhibition of MEK in 'oncogene-addicted' cancer cells, (driven by activated EGFR, HER2, ALK, MET and KRAS pathways) triggers the feedback activation of Stat3 through IL-6R and FGFR, leading to treatment resistance (Lee et al. 2014). In line with these results, Kim et al. showed that the constitutive activation of the IL-6/Stat3/NF κB pathway in $p53^{-}PTEN^{-}$ non-transformed MCF10A, was dependent on the proteolytic degradation of SOCS3 and generated highly metastatic and EMT-like CSCs. Thus, proteasoma inhibition restored SOCS3 protein levels and the selective IL-6R antagonist, tocilizumab, repressed the CSC compartments, hampered tumor growth and dissemination in vivo (Kim et al. 2014).

2.5 PARPi Affects CSC Survival

Recent breakthroughs displayed that inhibition of poly-ADP-ribose polymerase (PARP) could be a promising selective CSC-targeted therapy. Mechanistically, PARP is an abundant nuclear protein that mediates the repair of single strand breaks (SSBs) through base excision repair. The inhibition of PARP leads to the accumulation of SSBs that during replication are converted into double-strand breaks (DSBs), usually repaired by the homologous recombination (HR) pathway, mediated by BRCA1 or BRCA2 whereas in neoplastic cells with defective HR, the DSBs cannot be repaired and lead to cell death. It was shown that AZD2281, a PARP inhibitor (PARPi), preferentially targets glioblastoma stem cells (GSCs) and reduced their survival, expansion and tumor initiation capabilities, as well as having sensitized them to radiation therapy (Venere et al. 2014). Moreover, a PARPi, GPI 15427, was able to counteract GSC's resistance to temozolomide (Tentori et al. 2014). These examples opened a new road for the use of PARPi, even in the absence of mutations of *BRCA1/2*. This changed the classical idea of 'synthetic lethality' which exists between PARP and BRCA1/2 signaling pathways. Indeed, patients affected by triple negative breast cancer (non carriers of *BRCA1/2* mutations) have shown increased therapy response and survival following PARP inhibition (BSI-201) in combination with DNA-damaging chemotherapy. The latter of which may eventually obstruct the cellular DNA repair machinery and cause cell death (O'Shaughnessy et al. 2011). Moreover, deletions or mutations in other genes involved in key

genotoxic stress pathways such as *PTEN*, may sensitize them to PARPi administration (Mendes-Pereira et al. 2009). PARPi are currently under clinical evaluation in solid tumors as single agent or in combination with chemotherapy and detailed information about ongoing clinical trials has been published elsewhere (Curtin and Szabo 2013) (http://www.cancer.gov/clinicaltrials).

3 Stemness Modulator Drugs

Notwithstanding that CSCs embody a small portion of the tumor bulk, they are responsible for the heterogeneous cell population that constitutes the tumor mass and their intrinsic resistance to chemotherapy and radiotherapy shown by aggressive tumors. Indeed, CSCs possess both self-renewing capabilities, by means of generating two identical CSCs daughter cells through symmetrical division, and the ability to differentiate through asymmetrical division, yielding the multitude of cancerous cells that account for overwhelming tumor growth (Kreso and Dick 2014). As previously discussed, a prominent mechanism of therapeutic resistance includes an altered kinetic cell cycle in quiescent CSCs. They are spared by chemotherapy-induced cytotoxicity because they are not actively cycling cells but are capable of activating DNA repair mechanisms. Thus, forcing terminal differentiation of CSCs could be an extremely powerful weapon in preventing resistance and relapse. Ideally, a clinically effective response could be achieved by the simultaneous administration of anti-CSC therapy and conventional chemotherapy, in order to eliminate cytotoxic drug-susceptible non-CSCs and prevent their dedifferentiation in CSCs (Chaffer et al. 2011). Given that the development of clinical endpoints in this field may prove challenging, an emergent amount of stemness modulator drugs is already in clinical use and others are in preclinical or early stages of clinical evaluation. Some examples are listed below.

3.1 ATRA Induces Differentiation of CSCs

Among these, all-trans-retinoic acid (ATRA), a derivate of vitamin A, has already been demonstrated to be a potent differentiation-inducing drug and a successful treatment strategy, in combination with arsenic trioxide, for AML patients carrying the PML-RARα fusion protein (Zhou et al. 2005). Campos et al. (2010) reported that ATRA induced differentiation and radio- and chemo-sensitization of stem-like glioma cells. Given that, ALDH is a common marker of breast CSCs and a detoxifying enzyme responsible for the oxidation of intracellular aldehydes as well as of retinol to retinoic acid; it was shown that DEAB-mediated ALDH inhibition increased the CSC compartment by abrogating CSC differentiation. Conversely, ATRA treatment induced differentiation of breast CSCs and decreased the stem population (Ginestier et al. 2009). Similarly, Hammerle et al. (2013) suggested that

the neuroblastoma stem cells' response to 13-cis-retinoic acid (RA), could be enhanced by the proteasome inhibitor MG132. Interestingly, a combination of CSC genomics with connectivity map, analyzed a database of 6100 gene expression profiles of four breast cancer cell lines, treated with different concentrations of approximately 1000 FDA approved drugs. This revealed that ATRA is negatively associated with CSC-enriched gene expression signature. ATRA induced apoptosis, hampered mammosphere formation and forced differentiation of fulvestrant-resistant cells. Intriguingly, in the same study, a MEK inhibitor, selumetinib, sensitized the *K-RAS* mutant breast cancer cell line, which was enriched with CSCs, to the ATRA treatment (Bhat-Nakshatri et al. 2013).

3.2 SAHA Modulates Differentiation and Apoptosis of CSCs

Suberoylanilide hydroxamic acid (SAHA), also called vorinostat, a potent inhibitor of the histone deacetylase (HDAC) family, caused differentiation and apoptosis of several tumor type cells. In an in vivo prostate cancer tumor model, SAHA hampered tumor growth with low systemic toxicity (Butler et al. 2000). Additionally, HDAC inhibitors can be therapeutically exploited to specifically target slow cycling cells. For instance, SAHA, coupled with imatinib mesylate, successfully fostered apoptosis in quiescent chronic myelogenous leukemia stem cells and offered a novel strategy to overcome chemoresistance and the difficulties in targeting dormant cells (Zhang et al. 2010).

3.3 BMPs: An Actor of Balance Between Differentiation and Stemness

It is the general understanding that the bone morphogenic protein family (BMPs) is required to inhibit the stem cell state and mesenchymal traits in a variety of normal and cancerous epithelial tissues (Scheel et al. 2011; Cordenonsi et al. 2011) and promote differentiation of adult and pluripotent stem cells (Varga and Wrana 2005). Mechanistically, BMPs are members of the transforming growth factor-β (TGF-β) superfamily and bind to a combination of type I receptors (anaplastic lymphoma kinase 2 (Alk2), Alk3 (or BMPR1A), and Alk6 (or BMPR1B)) and type II receptors (BMPR2). They activate either the canonical BMP signaling pathway, through phosphorylation of smads receptors, or the PI3K/AKT-mediated non canonical BMP signaling pathway. Specifically, a BMP7 variant (BMP7v) abrogated in vitro proliferation of glioblastoma stem cells (GSCs) as well as the expression of stem associated markers and endothelial cord formation. In a glioblastoma orthotopic mouse model, BMP7v impaired tumor growth, invasion and angiogenesis (Tate et al. 2012). Likewise, our group demonstrated that BMP4 enhanced colorectal CSCs' differentiation and apoptosis and it their sensitized them to 5-fluorouracil

and oxaliplatin treatment. However, the *SMAD4*-defective tumors carrying either mutations in *PI3K* or loss of *PTEN* are refractory to the treatment mentioned above thus, confirming the BMP4-mediated activation of both canonical and non canonical pathways (Lombardo et al. 2011). On the contrary, molecules such as Coco, an antagonist of TGF-β ligands, reverse the effect of BMP thereby, enhancing the self-renewal of metastasis-initiating cells (Gao et al. 2012).

3.4 Resveratrol Affects CSC Self-Renewal

A number of epidemiological studies have proposed that resveratrol, a polyphenolic compound with which, many plant species are enriched with, exerts several biochemical activities associated with tumorigenesis such as, inhibition of inflammation, cell proliferation and angiogenesis as well as, sensitizing tumor cells to chemotherapy (Harikumar et al. 2010). Even though the influence of resveratrol on CSCs is still under evaluation, recent evidence showed that $KRAS^{G12D}$ mice, which spontaneously develop aggressive pancreatic cancer, treated with resveratrol developed smaller tumors (dimension and weight). Moreover, patient-derived pancreatic cancer and mice-derived $KRAS^{G12D}$ CSCs, lost their self-renewal capability in presence of resveratrol, possibly by the inhibition of Nanog, Sox-2, c-Myc and Oct4. In the same study, patient-derived CSCs underwent resveratrol-evoked apoptosis by activating caspase 3/7 and inhibiting XIAP and Bcl-2. Migration and invasion were suppressed following the inhibition of EMT related markers such as ZEB-1, SLUG and SNAIL (Shankar et al. 2011). Similarly, in Glioblastoma multiforme (GBM), resveratrol induced apoptosis and differentiation of stem-like cells and sensitized them to radiotherapy in vitro and in vivo, via disruption of STAT3 signaling (Yang et al. 2012). Thereafter, Sato et al. mechanistically explained the inhibitory effect observed after resveratrol treatment on self-renewal and the tumorigenicity of CSCs. Indeed, resveratrol promoted the phosphorylation and activation of p53, which in turn may directly favor Nanog degradation via proteasome machinery (Sato et al. 2013).

3.5 Cyclopamine Limits the Self-Renewal of CSCs

An additional plant-derived compound, the steroidal alkaloid cyclopamine, is a potent cancer preventing compound that directly binds to the heptahelical bundle of SMO (Chen et al. 2002). As already discussed in the present chapter, Hh signaling is essential for the maintenance of stem-like traits in multiple myeloma, leukemia and gastric cancer, among others (Peacock et al. 2007; Dierks et al. 2008; Song et al. 2011). Hh pathway inhibition through cyclopamine inhibited tumorsphere formation in vitro and the establishment of orthotopic glioblastoma tumors (Clement et al. 2007). The newly synthesized cyclopamine-derived inhibitor of the Hh pathway,

IPI-926, ameliorated cyclopamine characteristics such as oral bioavailability, higher metabolic stability, and a better pharmacokinetic profile (Tremblay et al. 2009). Cyclopamine and IPI-926 limited self-renewal potential of B-cell acute lymphocytic leukemia (B-ALL) cells (Lin et al. 2010). Interestingly, delivery of conventional chemotherapy, such as gemcitabine, to the tumor site, may be potentiated by the simultaneously administration of IPI-926. Indeed, in vivo inhibition of the Hh pathway increased intratumoral drug absorption in a gemcitabine-resistant pancreatic ductal adenocarcinoma model thus, making IPI-926 an important therapeutic strategy for the management of pancreatic cancer chemoresistance (Olive et al. 2009). IPI-926 is undergoing early step clinical trials for solid malignancy in combination with standard chemotherapy (Jimeno et al. 2013) (http://www.cancer.gov/clinicaltrials).

3.6 Curcumin Promotes CSC Differentiation

Curcumin (diferuloylmethane) derives from the Indian spice plant turmeric. Extensive preclinical studies showed its therapeutic potential in a variety of human diseases, including cancer. Due to its pleiotropic activities, curcumin is able to modulate a variety of normal or aberrant biological processes, hence it has been selected as a promising anti-cancer drug in several clinical trials (Gupta et al. 2013). Moreover, studies have shown that curcumin displayed capability of eliminating colon CSCs either alone or in combination with standard chemotherapy, such as FOLFOX (5-fluorouracil and oxaliplatin) and dasatinib (Nautiyal et al. 2011; Yu et al. 2009). Furthermore, Curcumin promotes GSCs terminal differentiation, which culminated in autophagy. Whereas, in an intracranial glioblastoma xenograft model, it repressed their self-renewal capability and tumorigenicity (Zhuang et al. 2012). Intriguingly, breast CSCs, derived from the MCF7 cell line, displayed inhibition of tumorsphere formation and the Wnt signaling pathway (Kakarala et al. 2010).

3.7 Metformin in CSC Biology

Metformin is a well-established oral anti-diabetic drug of the biguanide class. It is an agonist of the adenosine monophosphate-activated protein kinase (AMPK) and an inhibitor of PI3K, mTOR and IGF. It has gained attention for its in vitro and in vivo antitumor effects and is now being tested in several advanced clinical trials (Rattan et al. 2012) (http://www.cancer.gov/clinicaltrials). Metformin has also emerged as an important factor to counteract the retention of stemness and the activation of the EMT program of some cancer populations (Rattan et al. 2012). Metformin was able to inhibit the expression of Oct4 in the MCF7 cell line, mediated by 17-β-estradiol treatment, and to reduce the fraction of $CD44^{high}/CD24^{low}$

cells (Jung et al. 2011). In line with these results, Vazquez-Martin et al. observed that metformin deprived basal-like breast cancer cells of the stem compartment and suppressed an EMT program activation through the transcriptional repression of ZEB1, TWIST1, SNAI2 and TGF-β (Vazquez-Martin et al. 2010). Metformin depleted the CSC pool in both gemcitabine-sensitive and -resistant pancreatic cancer cells, by decreasing the expression of CSC-specific markers such as EpCAM, Notch, Nanog, and CD44, as well as reexpressing miRNAs, (e.g. let7a, let7b, miR-200b, and miR-200c) usually associated with cellular differentiation (Bao et al. 2012). The studies performed by Oliveras-Ferraros et al. attempted to anticipate the possible mechanisms of acquired resistance to metformin treatment. They observed that the potential of metastatic dissemination of breast stem-like cells seemed to be fueled by the chronic administration of metformin to the estrogen–dependent MCF7 cell line. Thus, the drug selected for the emergence of resistant cells, leads to a transcriptome reprogramming which, drives them towards a metastatic stem-like profile (Oliveras-Ferraros et al. 2014).

4 Microenvironment Modulator Drugs

4.1 Targeting the CSCs Vasculature Niche

There is proof of evidence that tumor-associated stroma and the extracellular matrix, are an extremely powerful source of herotypic signals, responsible for the activation of an EMT program on cancer cells and possibly to nurture the CSCs within their niche. Among the stromal compartment, endothelial cells play a major role in supporting the self-renewal capability of CSCs and in building up all the vasculature architecture needed from these cells to provide nutrients and an easy route to metastatic dissemination. While the contribution of endothelial cells to tumor angiogenesis is self-evident, our understanding on CSC survival and drug resistance is still incomplete. Pioneer work from Calabrese et al., showed how the formation of a vascular niche is directly involved in the function of CSCs. Interestingly, glioblastoma stem cells (GSCs) can be induced to differentiate in either endothelial cells or pericytes, as a consequence of their undifferentiated state and their strict dependence on microvasculature stimuli (Calabrese et al. 2007). Tumor vasculature is classically composed of a network of tortuous, saccular and extremely permeable vessels, endothelial cells that are abnormally covered by pericytes and an irregular basal membrane. As a result, cancer cells can easily penetrate into the bloodstream and colonize distant metastatic sites, and a higher interstitial hydrostatic pressure, due to plasma leakage, may impair the delivery of chemotherapeutic drugs to the tumor site (Jain 2005). Vascular endothelial growth factor (VEGF) was identified as an endothelial compartment mitogen which has a prominent role in positively regulating physiological and pathological angiogenesis. The mammalian VEGF family consists of five heparin-binding homodimeric glycoprotein of 45 kDa referred to as, VEGFA (VEGF), VEGFB, VEGFC, VEGFD and Placental growth factor (PlGF).

The predominant VEGF molecules are represented by several spliced variants denoted as, $VEGF_{121}$, $VEGF_{145}$, $VEGF_{148,}$ $VEGF_{165}$, $VEGF_{183,}$ $VEGF_{189}$, and $VEGF_{206}$ (Tischer et al. 1991). They are commonly secreted by macrophages, neutrophils, fibroblast and several cancer cells but not by endothelial cells themselves. VEGF receptors consist of VEGFR1 (FLT1), VEGFR2 (FLK1) and VEGFR3 (FLT4). VEGFR1 is able to bind VEGF, VEGFB and PlGF. VEGFR2 is activated by VEGF, VEGFC and VEGFD. Lastly, VEGFR3 is primarily involved in lymphangiogenesis as a receptor for VEGFC and VEGFD. Although all VEGFRs are tyrosine kinase receptors, VEGFR2, in response to VEGF stimulation, has captured the most attention as the predominant effector in cancer initiation and progression. This is explained by the fact that VEGFR1 binds VEGF with a higher affinity than VEGFR2 but conversely exhibits weaker tyrosine kinase activity in response to its ligand (Ellis and Hicklin 2008). In this context, Park et al. also proposed that VEGFR1 could act as a 'decoy' receptor able to negatively regulate VEGF activity, by preventing its binding to VEGFR2 (Park et al. 1994).

The binding of VEGFs to their cognate receptors induces dimerization and autophosphorylation of the intrinsic receptor's tyrosine residues and consequently activates the dominant PI3K-AKT, MAPK and FAK pathways. It is now well established that VEGFs and VEGFRs are expressed in a variety of tumors (including colon, breast, lung, prostate, and ovarian cancer). VEGF signaling interferes in cancer biology and interestingly in CSC function, independently of angiogenesis and in autocrine fashion. Conversely, it is popular belief that tumors rely on the classical paracrine VEGF-mediated sprouting angiogenesis, the increased permeability and the influence from the immune cells and the tumor microenvironment's fibroblasts (Goel and Mercurio 2013). The realization that VEGF signaling is a crucial determinant in EMT-induced cancer stemness, is becoming an emerging theme. Indeed, VEGF-mediated angiogenesis by itself is not sufficient but required to increase tumor initiating capacity and dissemination of breast cancer cells undergoing EMT, also suggesting that additional factors from the microenvironment are required (Fantozzi et al. 2014). For instance, a fraction of $CD133^{+}$ GSCs showed a 10–20-fold increase of VEGF secretion and displayed strongly angiogenic and hemorrhagic tumors through the enhancement of resident endothelial cell function and recruitment to the tumor site of bone marrow-derived endothelial progenitors (Bao et al. 2006). In murine models, GSCs may be induced to differentiate into endothelial cells and to directly contribute to tumor vasculature architecture, as proven by the positivity of those cells to VEGFR2 (Ricci-Vitiani et al. 2010). These findings clearly establish that VEGF, secreted by tumoral cells, acts as a paracrine factor to sustain angiogenesis and as an autocrine factor to boost cancer stemness.

Folkman (1971) was the first scientist to introduce the pioneer idea that solid neoplasms were always sustained by new vessel growth and envisioned angiogenesis as a new target for cancer treatment. In 2004, for the first time the FDA approved an anti angiogenic compound, called Bevacizumab, for clinical use in combination with standard chemotherapy. It is a humanized monoclonal antibody specific to

VEGF that prevents the interaction of VEGF to its receptor. It became the standard means of treatment for metastatic HER2 negative breast cancer, metastatic colorectal cancer, glioblastoma, advanced or metastatic non-small-cell lung cancer, advanced renal-cell carcinoma and recently, for persistent, recurrent, or metastatic cervical cancer (Tewari et al. 2014). Later, Aflibercept was approved as a 'decoy' receptor for VEGFA, VEGFB and PlGF (Patel and Sun 2014). The inhibition of VEGFR kinase activity, is another valid approach to counteract tumor angiogenesis. Sunitinib targets multiple receptor tyrosine kinases including PlGFR and VEGFRs in unresectable, local, advanced or metastatic disease in well differentiated pancreatic neuroendocrine tumors, renal-cell carcinomas, and imatinib-resistant gastrointestinal tumors. Similarly, Sorafenib inhibits Raf kinases, VEGFRs and PlGFR in thyroid, liver and hepatocellular carcinoma (Santoni et al. 2014). Since 1971, lots of studies have been published in the field and seemed promising but little efficacy has been shown yet. Besides their remarkable activity in the inhibition of primary tumor growth, anti-angiogenic drugs failed in producing lasting responses and patients' illnesses eventually progress (Bergers and Hanahan 2008). This could be partially explained by the fact that alternative adaptive resistance mechanisms, used to overcome the drug-mediated anti angiogenic effect, can occur. This could be the case when there is: an activation of alternative angiogenic pathways, including Fibroblast growth factor 1 (FGF1) and FGF2, Ephrin A1 (EFNA1) and EFNA2 and Angiopoietin1 (ANGPT1), the recruitment of proangiogenic cells, and the increased coverage of pericytes to support vessel integrity. Interestingly, in an in vivo engineered model of KRAS-driven pancreatic ductal adenocarcinoma, resistant to anti-VEGF therapy, the MEK inhibitor substantially decreased the release of granulocyte–colony stimulating factor (G-CSF) by the tumor cell, which is usually responsible for the recruitment and mobilization of pro-tumorigenic and pro-metastagenic $CD11b^+$ $Gr1^+$ myeloid-derived suppressor cells. $CD11b^+$ $Gr1^+$ cells also helped the establishment of metastases by secreting matrix metalloproteinases (MMPs) as well as the Bv8 molecule, endowed with pro angiogenic features. This study revealed that a combination of MEK inhibitor and anti-VEGF therapy substantially decreased tumor burden and angiogenesis (Phan et al. 2013). Likewise, anti angiogenic therapy eradicated the brain tumor stem cell niche in an in vivo model of c6 rat glioma cell line and enhanced the effect of the conventional cytotoxic agent, cyclophosphamide (Folkins et al. 2007).

Even upon anti-VEGF therapy, functional vessels tightly covered by pericytes have been observed. Indeed, endothelial cells can recruit perycites to protect themselves from anti angiogenic treatments and preserve their vascular structure. An attractive hypothesis suggested that $CXCR4^+$ GCSs were mobilized towards the tumor site through an SDF-1 gradient and, upon TGF-β release by endothelial cells, were forced to differentiate in pericytes and contributed to tumor vasculature and growth (Cheng et al. 2013). Moreover, Conley et al. showed that, hypoxic conditions limit the effectiveness of the antiangiogenic agents bevacizumab and sunitinib, by increasing breast CSC populations (Conley et al. 2012).

4.2 Therapeutic Implications of Neuropilins in CSCs Biology

VEGF receptors can functionally interact with other receptors and foster CSC-driven tumor growth and progression. Within the same context, Neuropilins (NRPs) were described earlier as neuronal receptors for the semaphoring family and also involved in axon guidance. They act as transmembrane glycoproteins with a short cytoplasmic domain that lacks intrinsic catalytic activity and function as co receptors of VEGFR1 and VEGFR2. NRP1 is commonly expressed by endothelial cells and tumor cells (Soker et al. 1998). Upon autocrine VEGF stimulation, NRP1 promotes stemness and renewal of VEGFR2$^+$ squamous skin CSCs (Beck et al. 2011). Similarly, viability, self-renewal and tumorigenicity of CD133$^+$ GSCs rely on autocrine VEGF/VEGFR2/NRP1 signaling and are maintained by a continuous secretion of VEGF (Hamerlik et al. 2012). Cao et al. showed that VEGF and NRP1 induced a dedifferentiated phenotype in vitro and promoted tumor formation in vivo (Cao et al. 2012). α6β1 integrin is necessary for the tumorigenicity of some subpopulations of breast CSCs and GSCs (Goel et al. 2014; Lathia et al. 2010). In triple negative breast cancers, NRP2 resulted preferentially expressed in breast CSCs and associated with α6β1 integrin. Upon VEGF stimulation of the NRP2- α6β1 complex, the focal adhesion kinase (FAK) mediated the activation of MAPK signaling and the subsequent expression of GLI1, an effector of the non canonical Hedgehog pathway. GLI1 in turn, induced BMI1 and positively fed back to the NRP2 expression, thus contributing to tumor initiation (Goel et al. 2013). NRP2 is also associated with aggressive prostate cancer and its expression is forced by PTEN loss. Activation of the VEGF/NRP2 axis culminates in BMI1 expression, which represses the transcription of the insulin like growth factor 1 receptor (IGF1R), commonly responsible for tumor progression. Interestingly, single targeting of NRP2 led to compensatory IGF-1R activation (Goel et al. 2012). Therefore, these findings offer a perfect example of how an ideal combination of conventional chemotherapy, stemness modulator drugs (in this case anti-NRP specific antibodies), and anti IGFR antibodies could reduce tumor bulk, overcome treatment resistance and prevent relapse (Fig. 16.2).

For instance, multiple compensatory signals could be activated when a single anti-angiogenic treatment is administrated, regardless of possible collateral stimulation of pathways involved in invasiveness or tumor cell stemness. Given that Bevacizumab does not inhibit VEGF binding to NRPs, Pan et al. (2007) generated two anti-NRP1 monoclonal antibodies specific to the binding site of semaphorin and VEGF on NRP1. This caused a reduction in cell proliferation as well as vascular density in a NSCLC in vivo model, assuming that the inhibition of NRP1, impairs vascular remodeling and thus rendering vasculature more responsive to anti VEGF treatment. In contrast with these findings, Snuderl et al. recently showed that the exclusive targeting of the PlGF/NRP1 pathway with the previously used phase I clinical trials, TB403 and 5D11D4, respectively an anti-murine PlGF antibody and an anti-human/murine PlGF antibody, reduced primary tumor burden and progression of medulloblastoma. PlGF seemed to be secreted by the tumor stroma,

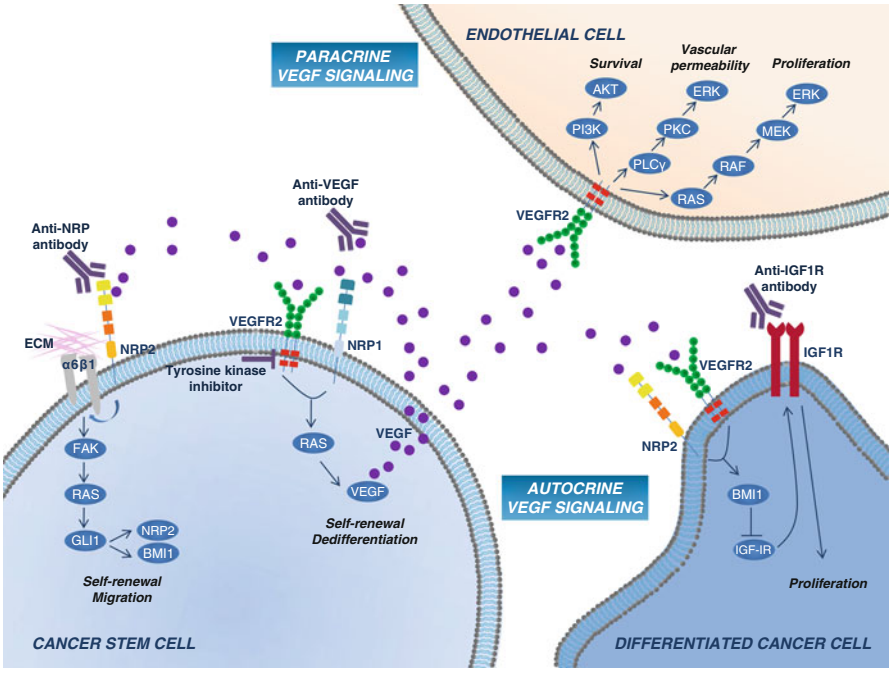

Fig. 16.2 *Therapeutic strategies to inhibit VEGF signaling in tumor cells*. Besides regulating the common paracrine pathway on endothelial cells to sustain angiogenesis, VEGF signaling, when potentiated by NRPs, exerts its role in the autocrine stimulation of CSC self-renewal and migration. NRP2 can also interact with α6β1 integrin and trigger the integrin-mediated activation of FAK signaling cascade that culminates in the induction of BMI1 and NRP2. NRP1 interaction with VEGFR2 promotes the release of VEGF in the extracellular compartment, sustaining both the autocrine loop and the paracrine endothelial cell activation. Inhibition of VEGF signaling can be achieved mainly by mAb targeting VEGF and small molecules TKIs. mAbs directed against NRPs have been developed and proved to hamper self-renewal and tumorigenic capabilities of CSCs. However, inhibition of NRP2 can lead to compensatory IGF1R expression via BMI1 down-regulation, supporting the importance of multiple therapy administration aimed at targeting both NRPs and IGF1R. Vascular endothelial growth factor (VEGF), Neuropilin (NRP), cancer stem cell (CSC), focal adhesion kinase (FAK), monoclonal antibody (mAb), tyrosine kinase inhibitor (TKI), insulin-like growth factor 1 receptor (IGF1R), extracellular matrix (ECM)

following tumor-derived Shh stimulation. PlGF only interacts with NRP1 rather than with VEGFR1 on medulloblastoma cells, for the enhancement of tumor spread. Authors suggested that the use of anti-NRP1 and –PlGF, in concert with standard chemotherapy, could make an additional improvement in the clinical setting (Snuderl et al. 2013).

Another example of multiple compensatory signaling activation was shown by Lu et al.. Indeed, bevacizumab treatment fostered an invasive phenotype in an in vivo model of GBM. The inhibition of VEGF suppressed the recruitment of the protein tyrosine phosphatase 1 B (PTP1B) from the VEGFR2/MET complex,

consequently restoring hepatocyte growth factor (HGF)-mediated MET phosphorylation and tumor invasiveness. Authors suggested that in selected patients with GBM, tumor recurrence could be avoided by the combined use of anti VEGF and anti MET treatments (Lu et al. 2012).

4.3 Targeting Microenvironment Stimuli

AMD3100 is an antagonist of CXCR4. This drug, in combination with G-CSF to improve hematopoietic stem cell mobilization to peripheral blood for autologous transplantation, was approved in 2008 by the FDA for clinical use as a treatment for non-Hodgkin's lymphoma and multiple myelomas (DiPersio et al. 2009b; DiPersio et al. 2009a).

Commonly used for leukemia in several clinical trials, AMD3100 prevents $CXCR4^+$ leukemia cell recruitment to the SDF-1-secreting bone marrow microenvironment, thus rendering cancerous cells more susceptible to cytotoxic drugs (Burger and Peled 2009). In agreement with this, invasive $CD133^+$ pancreatic CSCs expressed CXCR4 and predominantly metastasize in the liver, being attracted by a gradient of SDF1, which is secreted by the stroma compartment (Hermann et al. 2007).

Recently, CXCR4-SDF1 signaling has been identified as the driving force behind the establishment of bone metastasis in triple negative breast cancers. Particularly, CAF-rich stroma found in primary breast cancer secretes SDF-1 and IGF and selects tumor cell clones with high Src activity and thus, characterized by an activation of PI3K-AKT pathway. Src hyperactive clones were primed for bone metastasis because endowed with a greater chance of survival in the bone environment enriched with SDF-1 and IGF. Mechanistically, human mesenchymal stem cells were stimulated with a conditioned media from MDAMB231 cell line to constitutively secrete SDF-1 and IGF. Subsequently, authors cotransplanted breast cancer cell lines and stromal cells in an orthotopic mouse model. Following an in vivo treatment with CXCR4 inhibitor (AMD3100) and IGF1R inhibitor (BMS754807), the recovered cells were reimplanted and resulted in tumors, low in bone metastasis, compared to reimplanted cells from untreated tumors (Zhang et al. 2013).

Similarly, we recently showed that in colorectal cancer, the exposure to SDF1, HGF and OPN, increased the migratory capabilities of colorectal CSCs and induced the CD44v6 expression, an alternative splicing isoform of CD44, on transiently amplifying progenitors. Interestingly, in untreated colorectal CSCs, CD44v6 was already highly expressed whereas, it was lower in sphere-derived differentiated progeny and bulk primary cells. CD44v6 acts as a coreceptor of the tyrosine kinase receptor MET, and together with its ligand, the pleyotropic cytochine HGF, cooperates to promote survival and migration through the PI3K-AKT pathway. When blocking SDF-1-CXCR4 activity with AMD3100, it reduced the invasive potential and abrogated the CD44v6 expression induced by HGF and OPN. Similarly the PI3K inhibitor, BKM120, killed $CD44v6^+$ colorectal CSCs and impaired metastatic dissemination (Todaro et al. 2014). It is worth considering that targeting these

powerful effectors in the tumor microenvironment could have tremendous therapeutic implications. In this context, the use of compounds which, target both MET and HGF, are still under evaluation in several clinical trials (Peters and Adjei 2012) and only few of them were recently approved by the FDA. Although discovered as a MET tyrosine kinase inhibitor, Crizotinib was approved at the end of 2013 exclusively for the treatment of NSCLC as an ALK blocking compound (Malik et al. 2014). Similarly, Cabozantinib is a multi-kinase inhibitor against VEGFR1, 2 and 3, RET, MET, TIE-2 and KIT and is currently administered uniquely for progressive medullary thyroid cancer (Elisei et al. 2013). Clinical trials for prostate, brain, breast, and NSCLC are still undergoing (http://www.cancer.gov/clinicaltrials).

4.4 Hypoxia as a Therapeutic Target

Evidence that CD44 variant isoforms (CD44v) could promote survival and multidrug resistance has been shown by Ishimoto et al. In gastrointestinal cancer cells, CD44v enhanced the synthesis of reduced glutathione (GSH), the predominant intracellular antioxidant factor, by physically interacting with and stabilizing the cystine transporter subunit (xCT) at the plasma membrane. xCT is the light chain subunit of the cysteine-glutamate exchange transporter, which exchanges intracellular glutamate for extracellular cysteine, required for GSH synthesis. GSH protects the cell against reactive oxygen species (ROS) damages and suppresses $p38^{MAPK}$ activation, leading to cancer cell proliferation and resistance to ROS-inducing agents, such as docetaxel and cisplatin. As a result of these findings, in vivo exposure to sulfasalazine, a selective xCT inhibitor, induced $p38^{MAPK}$ signaling, enhanced response to chemotherapy, and avoided CD44-dependent tumor growth. Therefore, authors suggested that either sulfasalazine or CD44v-target therapy could abrogate ROS defense capabilities of CSCs and in turn sensitize to conventional cancer treatments (Ishimoto et al. 2011).

Normal stem cells as well as CSCs, harbor low levels of ROS and possess an efficient defense mechanism against oxidative stress (Diehn et al. 2009). An increase in ROS levels can occur in response to either environmental extrinsic (e.g. CAFs, CAMs, and hypoxia) or intrinsic oxidative stress (e.g. ROS producing enzyme and Jun D down-regulation), along with iron chelators, nitric oxide (NO), and genetic alterations in PTEN, von Hippel-Lindau (VHL), succinate dehydrogenase (SDH), RAS-MAPK, and PI3K-AKT accounts for the hypoxia-inducible factor 1 α (HIF-1α) stabilization (Moeller et al. 2004; Li et al. 2007; Lu and Kang 2010). As well as under normoxia, HIF-1α exerts its role in shielding tumor cells from oxygen deprivation and thus aids in meeting the metabolic requirements of the expanding tumor mass. The HIF family of transcription factors has a prominent role in a finely tuned and well characterized oxygen-sensor mechanism. They comprise a heterodimer of an oxygen dependent α–subunit (either HIF-1α, HIF-2α or HIF-3α) together with a constitutively expressed β-subunit (HIF-1β). Under normoxic conditions and

in presence of iron, prolyl hydroxylases (PDH) modifies Pro402 and Pro564 of HIF-1α and promotes the interaction with VHL, leading to ubiquitination and proteasomal degradation. It prevents HIF-1α to dimerize with HIF-1β and to bind with the coactivator CBP/p300 to the hypoxia response element (HRE) in the promoters of hypoxia-target genes, regulating proliferation/apoptosis, glycolysis, angiogenesis, and invasion/metastasis (Harris 2002). A high HIF-1α level is observed in many human cancers and is associated with poor prognosis in brain, breast, ovary, cervix, colorectal, prostate, bladder, and oropharynx cancers (Semenza 2003; Talks et al. 2000). Particularly, HIF-1α has been reported to be hyperactivated in TNBCs and necessary for the maintenance of the $CD44^{high}CD24^{low}$ cell population. Chen et al. identified XBP1, a component of the unfolded protein response (UPR) pathway, as a major controller of HIF-1α transcriptional activity in TNBCs. It is required for tumor relapse in a murine model and directly enriches the $CD44^{high}CD24^{low}$ population in vitro. XBP1 can also be associated with poor prognosis, suggesting that combinatory therapy using stem cell targeting drugs, such as inhibitors of the UPR pathway and standard chemotherapy may improve cancer therapeutic intervention (Chen et al. 2014).

A tight relationship exists between hypoxia and tumor dissemination. Low oxygen levels in tumor microenvironment promote the overexpression of EMT master regulators such as SNAIL, TWIST, and ZEB1, while it attenuates E-cadherin expression. Matrix remodeling requires basal membrane degradation via HIF-1α-dependent production of MMP2 and cathepsin D (CTSD). The so-called "invasive–switch" is guided by hypoxia and sustained by MET and lysyl oxidase (LOX) expression. Hypoxia facilitates both intravasation and extravasation of tumor cells through the increased production of VEGFA. Meanwhile, CXCR4, OPN, and Angiopoietin-like 4 (ANGPTL4) increase the chance of homing and outgrowth to secondary organs (Catalano et al. 2013).

HIF-2α also contributes to the hypoxia-driven "angiogenic-switch" and is directly linked to stem cell biology as a regulator of *OCT4* (Covello et al. 2006) and *c-MYC* (Gordan et al. 2007). Given that it displays a restricted tissue-specific expression pattern compared to its homologs, little attention has been given to addressing its pro angiogenic and pro tumorigenic features (Gordan et al. 2007). One key study showed the preferential expression of HIF-2α on GSCs compared to the differentiated and normal counterpart and its association with poor survival in glioblastoma patients. Authors underlined that HIF-2α may support the CSCs niche by providing survival and metabolic advantages through the modulation of *OCT4*, *GLUT1*, and *SERPINB9* expression. This suggests that new therapeutic approaches should be aimed at targeting stem cell specific molecules involved in neoangiogenesis (Li et al. 2009)

On the contrary, besides being a member of the HIF system, HIF-3α's role in the tumor hypoxia-inducible adaptive response system, is not well characterized. Indeed, it lacks the transactivation domain and likely functions as a negative regulator of HIF-1α and HIF-2α due to sequestration of HIF-1β (Kaur et al. 2005).

As previously discussed, preclinical data provide evidence that hypoxic tumor cells play a pivotal role in tumor progression and resistance to therapies. Moreover,

the pro metastatic effect elicited by angiogenesis-induced hypoxia can compromise clinical outcomes in patients. Thus, targeting intratumoral hypoxia can be considered the gold standard to be exploited in neoplastic malignancy. Nevertheless, it is clear that hypoxia is heterogeneously diffused within a given tumor cell population and is endowed with an even more differentiated extension among patient tumors. Based on this observation, an appropriate measuring of tumor hypoxia either by direct or indirect methods, will facilitate the selection of the patient's treatment as well as, the monitoring of their treatment-response (Wilson and Hay 2011). However, an interesting finding recently reported for the first time is that, a chemotherapeutic agent, in this case doxorubicin, can stabilize HIF-1α even in normoxic cells. Indeed, doxorubicin increased the expression of STAT1, with consequent stimulation of iNOS, intracellular synthesis of NO and HIF-1α accumulation (Cao et al. 2013).

In recent years, several drugs have been designed to selectively target chemo- and radio-resistant hypoxic cancer cells. According to the action mechanism, they could be tentatively categorized as (a) agents targeting HIF-1α DNA binding, (b) agents attenuating HIF-1α protein translation, (c) agents inducing HIF-1α protein degradation, (d) prodrugs inducing hypoxia-mediated cytotoxicity (e) HRE-driven expression of enzymes converting prodrugs and (f) agents targeting downstream HIF pathway effectors.

Specifically, HIF-1α function can be directly targeted via chetomin, a small molecule that precludes HIF-1α binding to the transcriptional coactivator p300/CBP (Kung et al. 2004). Similarly, the proteasome inhibitor bortezomib, which has been approved by the FDA for clinical use in multiple myeloma and mantle cell lymphoma patients refractory to at least one prior therapy, affects the C-terminal activation domain (CAD) of HIF-1α. It was shown that bortezomib enhanced the HIF-1α hydroxylation of Asn803 residue, by the dioxygenase factor-inhibiting hypoxia 1 (FIH-1), causing the inhibition of p300-HIF interaction (Kaluz et al. 2006). Intriguingly, anthracyclines, such as doxorubicin and daunorubicin, block HIF-1 binding to HRE sequence, providing new evidence in refining their use as antiangiogenic drugs (Lee et al. 2009).

HIF-1α expression can be modulated by the topoisomerase I inhibitor topotecan, one of the first hypoxia inhibitor ever tested on humans and currently approved for the treatment of small cell lung cancer and recurrent cervix carcinoma. Cardiac glycoside digoxin inhibited the translation of HIF-1α in an mTOR-independent manner. In preclinical settings, PX-478 appeared to inhibit HIF-1α mRNA expression and translation, and foster HIF-1α degradation by preventing its deubiquitination (Onnis et al. 2009). Contrasting data have been generated regarding the contribution of the mTOR pathway in the modulation of hypoxia. Several mTOR inhibitors, such as everolimus and temsirolimus, have been approved by the FDA for clinical use in renal cancer patients and displayed remarkable antiangiogenic activity and inhibition of HIF-1α (Del Bufalo et al. 2006). Hypoxia, especially in early stage tumors, may negatively regulate HIF-1α expression according to the intensity and duration of oxygen deprivation (Wouters and Koritzinsky 2008). Another indirect mechanism of HIF-1α inhibition includes the targeting of upstream

pathways (e.g. PI3K-AKT and RAS-MAPK) involved in HIF-1α protein translation (Poon et al. 2009). Interestingly, the tumor suppressor p53 mediates apoptosis under hypoxic conditions. However, cancer cells with dysregulated p53, escape programmed death and p53-mediated HIF-1α inhibition (Ravi et al. 2000). P53 may either interact with HIF-1α or mediate its degradation through HDM2 (Ravi et al. 2000) or compete with HIF-1α for p300 thus, blocking its transcriptional activity (Schmid et al. 2004). Agents targeting p53, aim at reactivating mutant p53. This is the case of RITA (reactivation of p53 and induction of cell apoptosis), which induces DNA damage in order to stimulate p53-evoked cell apoptosis and inhibits MDM2 to prevent p53 degradation. This mechanism seems to be hypoxia-independent (Yang et al. 2009).

HIF-1α degradation may be forced by the inhibition of chaperone HSP90. In normoxia and hypoxia, the HSP90 antagonists GA and 17-AAG mediate elimination of HIF-1α through E3 ubiquitin ligase and reduces angiogenesis in vivo (Isaacs et al. 2002). Trichostatin A is an inhibitor of HDAC and promotes proteasome-dependent HIF-1α degradation in osteosarcoma (Yang et al. 2006). Similarly, HDAC inhibitors FK228 and LAQ824 resulted in the abrogation of HIF-1α activity (Mie Lee et al. 2003; Qian et al. 2006). Of note, SAHA, the potent pan HDAC inhibitor, may act together with TRAIL, in breast cancer orthotopic models and down-regulate both VEGF and HIF-1α (Shankar et al. 2009).

One promising approach seeks to develop prodrugs that can be reduced by hypoxia in prodrug radicals, as intermediate products. In normoxia, they can be re-oxidized and converted back by oxygen while in hypoxic cells they can be either further reduced or fragmented so as to generate an active toxic drug. Examples of bioreactive prodrugs still in clinical development include RH-1, mitomycin C, AQ4N, PR-104, and SR4233. Some concerns have been reported regarding the prodrugs' penetration into poorly perfused tumors and their toxicity. The activation of aerobic reductase also in normal tissues or the additional generation of DNA reactive cytotoxins, make it hard to combine bioreductive prodrugs with standard chemotherapy (Wilson and Hay 2011).

Moreover, in tumoral cells prodrugs can be converted into cytotoxins by a hypoxia-regulated expression vector which, encodes the enzyme responsible for this reaction. Hypoxia targeted gene therapy has been tested in a preclinical setting and consists in the expression, in tumoral cells, of plasmid vector carrying genes driven by a promoter containing HRE and encoding: thymidine kinase (TK), cytosine deaminase (CD), uracil phosphoribosyltransferase (UPRT), and flavoprotein cytochrome c P450 reductase (CPR) (Patterson et al. 2002; Hsiao et al. 2014). A triple suicide gene therapy has proven to enhance cytotoxicity to ganciclovir and 5 fluorocytosine and sensitize colorectal cancer cells, both in vitro and in vivo, to radiotherapy by simultaneous expression of TK, CD and UPRT (Hsiao et al. 2014).

Finally, multiple agents also aim at targeting the downstream component of the HIF signaling pathway such as the LOX inhibitors, β-aminoproprionitrile (βAPN) or anti-LOX antibody, which binds the LOX active site and blocks its enzymatic function (Erler et al. 2009)

5 Challenges and Limitations of Targeting Cancer Stem Cells and Their Niche

Conventional anti-cancer drug development has been focused on the identification of cytotoxic chemotherapeutic agents that can target deregulated pathways and molecular markers in tumor cells. Despite all efforts, patients undergoing chemotherapy, after an apparent remission, often relapse and develop more aggressive diseases. This emphasizes that CSCs may be responsible for therapy failure due to the specific activated mechanisms which are peculiar to the undifferentiated status of these cells. In this context, novel compounds have been precisely designed to eliminate CSCs or affect their microenvironment and, administered in concert with conventional chemotherapy, can lead to tumor bulk shrinkage and ablate resistance and relapse. Of note, there is a need to refine such therapies to counteract their side effects. Indeed, such approaches could impair normal stem cell niches, have 'off target' effects on signals required for normal cells survival or, and as well as standard treatments, they should be administered at concentrations harmless to patients.

6 Conclusion and Future Perspectives

The reviewed data show only a partial portion of the existing therapies in the field. Anyhow, they seek to emphasize that despite of the efforts that have been made to develop powerful CSCs targeted therapy, multiple obstacles still need to be faced for the achievement of long lasting clinical benefits. The future use of appropriate tumor models and technologies reflecting the phenotypic, genetic and epigenetic tumor heterogeneity constantly evolving to counteract the hostile milieu, will possibly overcome the achieved disappointing results. Moreover, a multitude of new inhibitors are currently being investigated and will possibly conduct to some encouraging experimental evidence.

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Chapter 17
Cancer Stem Cells and Chemoresistance: Strategies to Overcome Therapeutic Resistance

Margaret L. Thomas, Krysta M. Coyle, Mohammad Sultan, and Paola Marcato

Abstract Cancer stem cells (CSCs) are hypothesized to initiate cancer and give rise to heterogeneous tumors made up of self-renewing CSCs and the differentiated, less tumorigenic non-CSCs, which make up the bulk of the tumor. Importantly, in terms of successful patient treatment, CSCs are also more resistant to commonly used chemotherapeutics. Multiple mechanisms have been identified for CSC-associated chemoresistance. These mechanisms include increased expression of ABC transporter efflux pumps, aldehyde dehydrogenase (ALDH) detoxification enzymes, anti-apoptosis proteins, enhanced DNA repair mechanisms, increased activation of the embryonic signaling pathways (Notch, Wnt and Hedgehog), and quiescence. Identification of these mechanisms has led to development of specific strategies to circumvent CSC-associated chemoresistance (e.g. inhibitors of ABC transporters, ALDH enzymes, and Notch, Wnt, and Hedgehog pathways, and epigenetic modifying drugs). Future clinical evidence will reveal if employing these adjuvant therapies will eradicate CSCs along with the bulk of the tumor, and lead to improved patient outcomes with decreased cancer recurrence.

Keywords Cancer stem cells • Chemotherapy resistance • Drug efflux • Detoxification • DNA repair • Chemotherapeutics

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1 Introduction

1.1 Chemotherapy Resistance and Cancer

Cancer is comprised of many distinct diseases with diverse mechanisms of initiation, metastatic potential, treatment strategies and expected patient outcomes. Tumors are classified as distinct subtypes based on physiological and histopathological assessments, expression of certain biomarkers, and mutations. This information is then used clinically in the application of the most appropriate therapies. For example, breast cancers can be classified as one of at least four different subtypes (luminal A, luminal B, basal-like, Her-2 positive) with distinct prognoses and treatment recommendations (Carlson et al. 2009; McSherry et al. 2007). A breast cancer classified as luminal B is a higher grade tumor (poorly differentiated, with aggressive tendencies) that expresses estrogen receptor (ER). This cancer would typically be treated by surgical resection, an adjuvant chemotherapy that targets highly proliferative cells (Fig. 17.1) and a targeted hormone therapy (e.g. ER antagonist tamoxifen). Such strategies are improving patient outcomes (Berry et al. 2005). For breast cancer, patient survival has risen from 35% to over 75% in the last 50 years. Unfortunately, in the vast majority of patients that eventually succumb to the disease, it is not due to the primary tumor, but to recurrent metastatic disease which is typically resistant to chemotherapy. After the initial treatment success for the primary tumor, treatment of the recurrent disease is met with reduced success even when treated with other chemotherapeutic drugs.

In treating all cancers, overcoming therapy resistance and recurrence after remission is a major challenge. Chemotherapeutic resistance can either be an innate characteristic of the primary tumor or developed later during recurrence (acquired resistance). Furthermore, chemoresistance is a complex problem as it is not usually isolated to one specific subclass of drug, but tends to include multiple drug classes. Multidrug resistance (MDR) is a major hindrance to improving patient survival in all cancers. Perhaps an even greater concern, which current clinical strategies are only beginning to consider, is the intratumoral heterogeneity that exists within individuals and the potentially important role that this plays in dictating therapy resistance and recurrence (Burrell and Swanton 2014). Intratumoral diversity is at the genomic, epigenomic, transcript and proteomic level and the clonal evolution that occurs during the course of the disease and treatment is only partially understood.

1.2 Chemotherapy Resistance in Cancer Stem Cells

Increasing evidence suggests that within a tumor there exists cancer cells with varying abilities to both initiate tumors and metastasize (Al-Hajj et al. 2003; Bonnet and Dick 1997; Carpentino et al. 2009; Charafe-Jauffret et al. 2009, 2010; Choi et al.

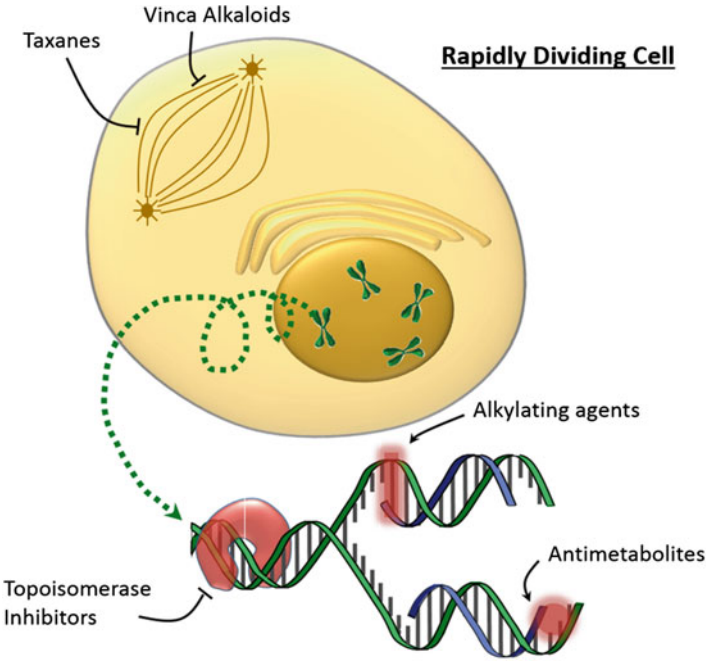

Fig. 17.1 Mechanisms of action of conventional chemotherapies such as taxanes, vinca alkaloids, alkylating agents, antimetabolites, and topoisomerase inhibitors, which target rapidly dividing cells. Taxanes disrupt proper assembly of microtubules, effectively inhibiting mitosis. For example, paclitaxel and docetaxel promote the irreversible assembly of tubulin into microtubules; unable to disassemble their microtubules, cells will then undergo apoptosis (Wang et al. 2011). Vinca alkaloids (e.g. vinblastine) are also antimitotic agents and these molecules interact with tubulin to prevent its polymerization into microtubules and thus prevent cell division (Ngo et al. 2012). Alkylating agents (e.g. cyclophosphamide, cisplatin, temozolomide) transfer alkyl carbon groups onto a variety of biological molecules including DNA (Fu et al. 2012). These agents form covalent adducts in the DNA by reacting with the ring nitrogens and extracyclic oxygen atoms of DNA bases; most chemotherapeutic alkylating agents modify both the nitrogens and oxygen groups. Failed DNA replication and breaks in the strands will induce apoptosis. Antimetabolites (e.g. 5-fluorouracil) have similar structures to nucleosides and are able to inhibit the enzymes required for DNA synthesis, or become incorporated into the DNA and induce apoptosis (Khan et al. 2012). Topoisomerase I and II inhibitors (e.g. doxorubicin, daunorubicin, mitoxantrone) cause the accumulation of single-strand or double-strand breaks respectively through stalling the topoisomerase enzymes at their intermediate enzyme-DNA complex state (Bailly 2012). The topoisomerases are required in DNA synthesis as they navigate the unwinding of condensed DNA by inducing single or double-stranded breaks; when topoisomerase inhibitors are added and these breaks accumulate, the cell will initiate apoptosis

2009; Coyle and Marcato 2013; Dalerba et al. 2007; Ginestier et al. 2007). The most tumorigenic cancer cells, the cancer stem cells (CSCs), are a subpopulation of tumor cells hypothesized to be largely responsible for the gene expression heterogeneity that exists within tumors. CSCs have unlimited renewal potential, and give rise to "differentiated" cancer cells with limited renewal potential and decreased

tumorigenicity. However, when considering the treatment success of patients, it is the relative resistance of CSCs to many standard chemotherapeutics that is of most concern (Fig. 17.2) (Dylla et al. 2008; Eramo et al. 2006; Gong et al. 2011; Hirschmann-Jax et al. 2004; Hu et al. 2012; Kucerova et al. 2014; Liu et al. 2006; Marcato et al. 2009; Tanei et al. 2009; Thomas et al. 2014; Tomuleasa et al. 2010; Touil et al. 2014). There are several potential mechanisms for the resistance of CSCs to these chemotherapies, such as increased DNA damage response, deregulation of apoptosis pathways, increased efflux transporter expression and increased expression of drug detoxification enzymes (Fig. 17.3). This chapter will overview what is currently known about the mechanisms of chemoresistance in CSCs, will discuss strategic therapies to circumvent this resistance, and will highlight the importance of understanding and avoiding CSC drug resistance.

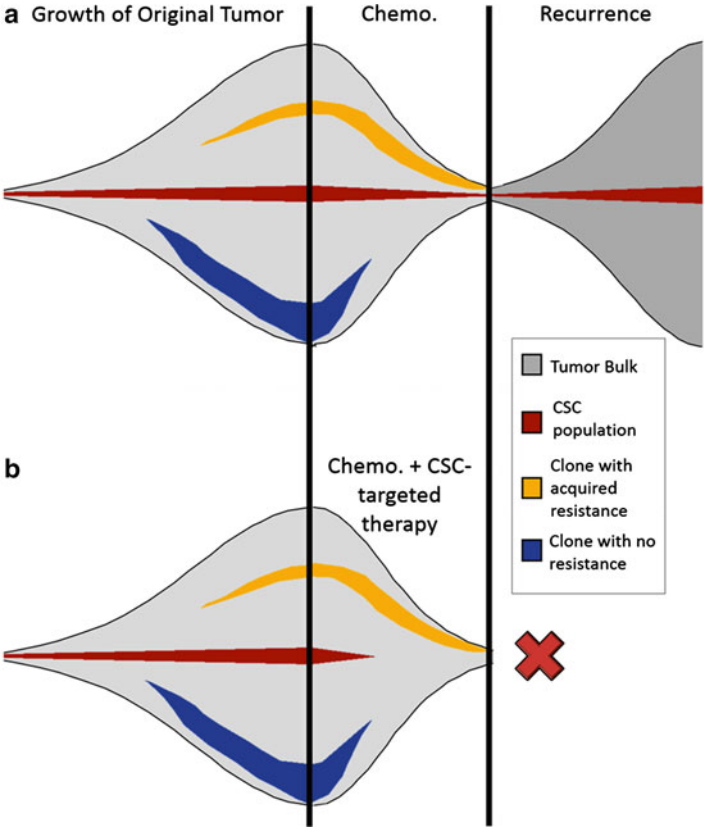

Fig. 17.2 Model of cancer stem cell-associated chemotherapy resistance and recurrence. (**a**) Expansion of original tumor based on the CSC population with development of multiple clones within the tumor. Chemotherapy reduces tumor bulk and eliminates chemosensitive clones but does not remove CSCs or chemoresistant clones; it is the CSC population that is then responsible for tumor recurrence. (**b**) The addition of CSC-targeted therapy to the chemotherapeutic regime eliminates the CSC population, and though a drug-resistant clone persists through treatment, it is not able to induce tumor recurrence without CSCs.

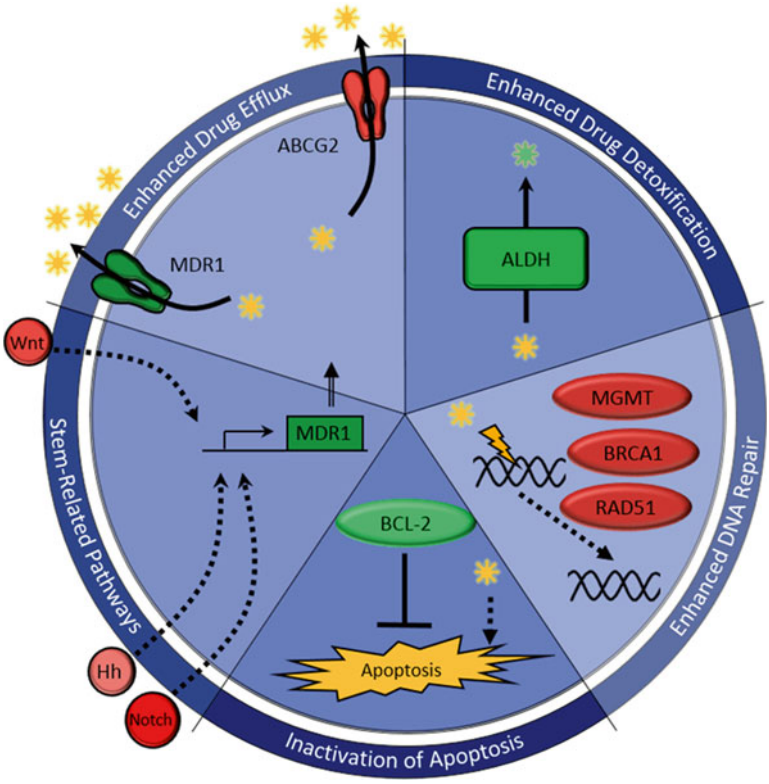

Fig. 17.3 Mechanisms of CSC-associated chemotherapy resistance. Multiple mechanisms of CSC-associated chemotherapy resistance mechanisms have been identified and most are shown in clockwise order: (1) Increased expression of ABC transporters including MDR1/ABCB1 and BCRP/ABCG2 leads to increased efflux of many subclasses of chemotherapeutic and multidrug resistance. (2) Increased expression of ALDH enzymes (detected by the Aldefluor assay) leads to detoxification and inactivation of chemotherapeutics (e.g. cyclophosphamide). (3) Enhanced DNA repair due to increased expression of DNA repair proteins such as MGMT, BRCA1, and RAD51. (4) Reduced apoptosis due increased levels of anti-apoptotic protein BCL-2. (5) Increased activation of embryonic signaling pathways Wnt, Notch and Hedgehog leads to chemoresistance via altered signaling (e.g. increase expression of drug efflux proteins such as MDR1). This illustrates the cross-talk that exists between the chemoresistance mechanisms. Other mechanisms not shown here include quiescence and epigenetic changes but are discussed in the chapter.

2 Chemoresistance of Cancer Stem Cells by Enhanced Drug Efflux Mechanisms

2.1 Enhanced Efflux Mechanisms of Cancer Stem Cells

In addition to cell surface marker staining, one commonly used method for isolating CSCs of various cancers is the exclusion of cellular stains such as Hoechst stain (Ponti et al. 2005). Relative to less tumorigenic non-CSCs, CSCs have decreased

staining capacity for cell-permeable dyes and they can be identified and isolated by fluorescence activated cells sorting (FACS) based on this differential staining. CSCs identified by this method are often referred to as the side population (SP) (Hadnagy et al. 2006). The decreased staining of CSCs is not due to decreased intake of the stain, but rather is due to increased efflux of the stain. Like normal stem cells, CSCs have enhanced efflux mechanisms, which in many cases is due to increased expression of ATP-binding cassette proteins or ABC transporters (Bunting 2002). In particular, the efflux capacity of the SP has been attributed to increased expressed of ABCB1, ABCC1, ABCG2, and ABCA3 (Chiba et al. 2006; Haraguchi et al. 2006; Hirschmann-Jax et al. 2004; Kim et al. 2002; Loebinger et al. 2008; Zhu et al. 2010). These transporters are also known to efflux chemotherapeutic drugs and are a common cause of chemotherapy resistance (Gottesman et al. 2002). Therefore, increased expression of ABC transporters by CSCs is a primary mechanism of chemotherapeutic resistance, encompassing all cell-permeable drugs. Given the strong association between ABC transporter expression and CSCs, it is not surprising that their expression is sometimes used to identify CSCs (Keshet et al. 2008; Schatton et al. 2008).

2.2 ABC Transporters and Chemoresistance in Cancer

ABC transporters are part of a large family of evolutionarily conserved proteins found in both prokaryotes and eukaryotes. In humans there are 48 known ABC transporters, divided into seven subfamilies. These multisubunit, ATP-powered transmembrane proteins function in the transport of substrates across membranes and can either be classified as importers or exporters. For extensive overviews of these proteins please see the following reviews; Hollenstein et al. 2007; Rice et al. 2014; Schinkel and Jonker 2003; Sosnik 2013. ABC importers (class 1 and 2) function in nutrient uptake, while ABC exporters efflux drugs (e.g. chemotherapeutics, antibiotics), peptides and toxins, and are involved in glycolipid flipping. Therefore, in addition to potentially causing problems in the effective treatment of cancer, these exporters also cause wide-spread anti-bacterial drug resistance. ABC exporters are able to efflux a wide array of chemotherapeutics, across multiple drug classes (e.g. colchicine, doxorubicin, etoposide, vinblastine, and paclitaxel) and are often responsible for MDR in cancer (Figs. 17.1 and 17.3).

One well-studied ABC exporter, ABCB1 (also known as P-glycoprotein or MDR1) is often implicated in chemotherapy resistance in many cancers, including gastrointestinal cancers and acute myeloid leukemia (AML) (Shaffer et al. 2012). In 1989, Goldstein et al. performed a large-scale cross-cancer comparative study and classified tumors based on their MDR1 expression. They reported that intrinsically chemotherapy-resistant colon, liver, kidney and pancreatic cancers, as well as some carcinoid tumors, chronic myeloid leukemia (CML), and non-small cell lung cancers expressed the highest levels of MDR1 (Goldstein et al. 1989). For breast cancer, MDR1 expression is varied. Meta-analyses suggest that MDR1 may be

expressed in 40 % (Trock et al. 1997), or as high as 66 % of breast cancers (Larkin et al. 2004). Furthermore, some evidence suggests that chemotherapy treatment may increase expression of MDR1, which may explain why at least some acquired resistance in breast cancer correlates with increased MDR1 expression following neoadjuvant chemotherapy treatment (Lizard-Nacol et al. 1999; Rudas et al. 2003). Patients with high levels of MDR1 are three times more likely to be non-responsive to chemotherapy than MDR1 negative patients (Trock et al. 1997). Finally, in direct proof of the role of MDR1 in chemotherapy resistance, inhibition of MDR1 expression sensitized breast cancer cells to chemotherapeutics (Jin et al. 2010).

Additionally, other ABC transporters often linked to chemotherapeutic resistance include ABCG2 (Mao and Unadkat 2014). This transporter is also commonly referred to as the breast cancer resistance protein (BCRP) due its first identification in a drug-resistant clone of MCF7 breast cancer cells; BCRP imparted resistance to multiple chemotherapeutics in the cell line (e.g. mitoxantrone, doxorubicin, and daunorubicin) (Doyle et al. 1998). ABCG2/BCRP expression is associated with chemotherapy resistance in AML patients (van den Heuvel-Eibrink et al. 2002). In breast cancer patients, neoadjuvant chemotherapy increased ABCG2/BCRP expression and its expression correlated with decreased disease-free survival (Kim et al. 2013), illustrating the importance of the exporter to chemoresistance.

2.3 Chemoresistance of Cancer Stem Cells and Increased ABC Transporter Expression

There is increasing evidence linking the chemoresistance of CSCs specifically to MDR1 expression (Fig. 17.3). Pancreatic CSCs identified in Panc-1, HPAC, and CFPAC-1 cells were resistant to gemcitabine and expressed high levels of MDR1 (Hong et al. 2009; Wang et al. 2013). $CD133^+$-identified prostate CSCs were enriched for MDR1 expression (Rentala and Mangamoori 2010). Ovarian CSCs identified by cell surface markers $CD44^+CD117^+CD133^+$ express high levels of MDR1 (Fong and Kakar 2010). A glioblastoma CSC cell line exhibited increased MDR1 expression and resistance to doxorubicin, etoposide, and carboplatin (Nakai et al. 2009). In $CD34^+CD38^-$-identified AML CSCs, MDR1 expression was elevated and they were resistant to daunorubicin (Ho et al. 2008). Breast cancer cell lines enriched for CSCs and spheroids had significantly higher MDR1 expression and were resistant to multiple chemotherapeutics, including doxorubicin, cisplatin, and etoposide (Wright et al. 2008).

In addition to MDR1/ABCB1, other ABC transporters are preferentially expressed in sub-populations of cancer cells identified as CSCs or having CSC-like properties. For example, in $CD34^+CD38^-$-identified AML CSCs, BCRP/ABCG2 is overexpressed and the cells are resistant to mitoxantrone; however, inhibition of BCRP was insufficient to sensitize the cells to the drug (Raaijmakers et al. 2005). This is likely due to the redundancy of ABC transporters – blocking one specific

exporter specifically is often insufficient to significantly abrogate ABC-mediated drug efflux, since often multiple transporters are overexpressed. In agreement with this, AML CSCs express higher levels of both MDR1 and BCRP (Ho et al. 2008). Esophageal carcinoma CSCs express increased ACBG2/BCRP and ABCA5 (Huang et al. 2009a). Head and neck CSCs have higher ABCG2 levels and increased resistance to cisplatin, 5-fluorouracil, paclitaxel, and docetaxel (Lim et al. 2011). SP-identified neuroblastoma CSCs had high levels of ABCG2/BCRP and ABCA3 expression and were resistant to mitoxantrone (Hirschmann-Jax et al. 2004). In prostate CSCs, chemoresistance was dependent upon ABCC1 expression, which was regulated by Notch signaling (Liu et al. 2014a). Analysis of recurrent prostate cancers revealed coinciding increased expression of ABCG2 and stemness markers SOX2, OCT4, and KLF4 (Guzel et al. 2014). Therefore, in these and other cancers, there is a strong association between expression of drug resistance-associated ABC transporters and CSCs; later, in Sect. 8.2, we discuss the evidence for targeting CSCs with anti-ABC transporter inhibitors.

3 Chemoresistance of Cancer Stem Cells Due to Aldehyde Dehydrogenase-Mediated Detoxification

3.1 Identification of Cancer Stem Cells by Increased Aldehyde Dehydrogenase Activity

Other than the various cell surface markers, the most common method used to identify CSCs is based on high cytoplasmic aldehyde dehydrogenase activity (ALDH). This activity is intrinsic to the CSCs of many cancers. Specifically, ALDH activity is measured employing a fluorescence-based enzymatic assay combined with FACS (the Aldefluor assay). The Aldefluor assay measures conversion of a membrane permeable ALDH substrate, BODIPY aminoacetaldehyde, to a fluorescent, cytoplasmic-retained product, BODIPY aminoacetate. This assay was originally developed for isolation of viable hematopoietic stem cells from human umbilical cord blood (Storms et al. 1999). However, following the publication of two seminal papers in 2007 that showed that Aldefluor$^+$-isolated cancer cells of breast tumors and leukemias had CSC qualities (i.e. increased tumorigenicity and give rise to heterogeneous tumors) (Cheung et al. 2007; Ginestier et al. 2007), the use of this assay was re-purposed for CSC identification, isolation and study. Aldefluor$^+$-identified CSCs have been reported in many tumor types, including the cancers of liver, head and neck, lung, pancreas, cervix, thyroid, prostate, colon, bladder, and ovaries (Basak et al. 2009; Boonyaratanakornkit et al. 2010; Bortolomai et al. 2010; Carpentino et al. 2009; Chu et al. 2009; Clay et al. 2010; Deng et al. 2010; Huang et al. 2009b; Jiang et al. 2009; Li et al. 2010; Ma et al. 2008a; Rasheed et al. 2010; Rasper et al. 2010; Su et al. 2010; Todaro et al. 2010; Ucar et al. 2009; van den Hoogen et al. 2010; Wang et al. 2010).

3.2 Function of Aldehyde Dehydrogenases in Detoxification

ALDHs are a superfamily of enzymes that catalyze the oxidation of aldehydes to carboxylic acids. There are 19 genetically distinct isoforms expressed in humans (Marchitti et al. 2008). The ALDH enzymes have tissue and organ-specific expression profiles. Similarly, expression of some ALDH isoforms is associated with certain cancers (Marcato et al. 2011a). In general, most ALDH enzymes function in the removal of toxic aldehydes generated during metabolic processes (Marchitti et al. 2008). This detoxification activity implies a potential function in the resistance of certain chemotherapeutics. Aldehydes are naturally occurring compounds that are formed by the metabolism of carbohydrates, lipids, vitamins, amino acids, and steroids; aldehydes will react with thiol and amino groups and lead to cellular damage. ALDHs oxidise and effectively detoxify many reactive aldehydes to protect cells. Furthermore, this detoxification activity extends beyond the reactive aldehydes generated from metabolic processes to aldehydes of exogenous origin, such as the metabolites of alcohol and chemotherapeutics.

3.3 Aldehyde Dehydrogenases and Cyclophosphamide Resistance

Biotransformation of some anti-cancer drugs generates reactive aldehydes, which in addition to their primary mode of action contributes to their toxicity. One example is the commonly used chemotherapeutic cyclophosphamide (Figs. 17.1 and 17.3). This alkylating agent and DNA synthesis inhibitor is used to treat many cancers such as breast, lung, ovarian cancer, as well as AML, CML, neuroblastoma, sarcomas, and lymphoma (Bonadonna et al. 1995; Di et al. 1990; Luce et al. 1971; Socie et al. 2001; Thurman et al. 1964). Cyclophosphamide is a pro-drug that is converted to its main active metabolite, 4-hydroxycyclophosphamide, by liver enzymes. 4-hydroxycyclophosphamide exists in equilibrium with its tautomer, aldophosphamide, an aldehyde and ALDH substrate (Emadi et al. 2009; Hilton 1984; Jones et al. 1995; Russo and Hilton 1988). ALDHs oxidize aldophosphamide and generate the inactive metabolite carboxycyclophosphamide (Fig. 17.3).

Prior to the association of ALDH activity with CSCs, ALDH enzymes were known to inactivate cyclophosphamide and this was seen as desirable activity since it limited the toxicity of the chemotherapeutic (i.e. ALDH expression is high in bone marrow stem cells, liver cells and intestinal cells) (Hilton 1984; Jones et al. 1995; Russo and Hilton 1988). At the time, the potential resistance of a subpopulation of tumor cells (i.e. CSCs with high ALDH activity) was not a major concern. Studies on cyclophophamide resistance mechanisms have identified the ALDH1A1 isoform, and the ALDH3A1 isoform to a lesser extent, as being primarily responsible for detoxifying cyclophosphamide (Magni et al. 1996; Moreb et al. 2007; Sladek et al. 2002). When expression of ALDH1A1 was induced in L1210

cells, the cells became more resistant to cyclophosphamide (Magni et al. 1996). ALDH1A1-deficiency in mice resulted in the hematopoietic cells having increased sensitivity to cyclophosphamide (Levi et al. 2008). In breast cancer patient tumors, ALDH1A1 expression was predictive of tumor responsiveness to cyclophosphamide treatment (Sladek et al. 2002). Conversely, when ALDH1A1 and ALDH3A1 expression was reduced by RNA interference (RNAi), there was an increase in cyclophosphamide toxicity to lung adenocarcinoma cell line A549 (Moreb et al. 2007). Together, these experiments suggest that ALDH1A1 and ALDH3A1 are involved in cyclophosphamide resistance in multiple cancer types. Therefore, which ALDH isoforms are expressed in and used to identify CSCs of various cancers, becomes important when considering the potential role of ALDH in CSC chemotherapeutic resistance.

3.4 Specific Aldehyde Dehydrogenase Isoforms Associated with Cancer Stem Cells of Different Cancers

When first used to study breast CSCs, the Aldefluor assay was believed to be specific for one particular ALDH isoform found in high abundance in hematopoietic stem cells, the ALDH1A1 isoform. However, while the 19 ALDH isoforms do have preferred substrate specificity, they also have cross-reactivity, making it likely that the Aldefluor assay can detect the activity of multiple ALDH isoforms (Marcato et al. 2011b; Marchitti et al. 2008). In agreement with this, a number of ALDH isoforms have been implicated as being responsible for the high Aldefluor/ALDH activity associated with CSCs, with certain isoforms being associated with specific cancers (Marcato et al. 2014). Van den Hoogen et al. used the Aldefluor assay to identify prostate CSCs, and reportedly found low expression of ALDH1A1, but higher expression of ALDH7A1 in prostate cancer cells and tissues (van den Hoogen et al. 2010). This raises the possibility that for prostate cancer ALDH7A1 may be contributing to the Aldefluor activity of these cells. For colon cancer, it was reported that 98 % of colon cancer samples were positive for ALDH1B1 expression, leading the authors to propose that ALDH1B1 may contribute to Aldefluor activity in colon cancer (Chen et al. 2011). In another colon cancer study, murine xenograft tumors from colorectal cancer cell lines were investigated for ALDH gene expression, and ALDH3A1, ALDH5A1, and ALDH1A1 were expressed more in the tumorigenic populations than in non-tumorigenic cells (Dylla et al. 2008). However, of the three ALDH isoforms, the mRNA for ALDH1A1 was expressed at higher levels than the other two enzymes. In liver cancer cell lines, it was confirmed by both quantitative PCR and western blotting that expression of ALDH1A1 was increased in the population of $CD133^{+}$-identified CSCs (Ma et al. 2008a). In ovarian cancer, ALDH1A1 expression has been clearly implicated in Aldefluor activity. Mice with xenograft ovarian tumors were treated with nanoliposomes that silenced ALDH1A1 expression, and Aldefluor analysis showed that silencing ALDH1A1 resulted in significantly lower Aldefluor activity (Landen et al. 2010), and its expression correlated with more aggressive disease in patients (Liebscher et al. 2013).

A few studies suggest that ALDH1A3 expression may be at least as an important as ALDH1A1 in influencing the Aldefluor activity of cancer cells and CSCs. For breast cancer, gene expression and knockdown studies revealed that ALDH1A3 expression was the primary isoform contributing to Aldefluor activity of breast cancer patient tumors and cell lines (Marcato et al. 2011b). Later, similar studies performed in melanoma implicated both ALDH1A1 and ALDH1A3 expression as being important in determining Aldefluor activity in melanoma (Luo et al. 2012). Furthermore, the authors conducted knockdown studies in melanoma cells and found that ALDH1A3 expression contributed to their tumorigenicity. More recently, it was demonstrated that for mesenchymal glioma and lung CSCs Aldefluor positivity was associated with enriched ALDH1A3 expression (Mao et al. 2013; Shao et al. 2014).

3.5 Aldehyde Dehydrogenase Expression by Cancer Stem Cells Imparts Resistance to Multiple Chemotherapies

There is evidence suggesting that Aldefluor$^+$-identified CSCs are more resistant to cyclophosphamide. CSC enrichment was observed in colorectal cancer xenograft tumors after cyclophosphamide treatment, and this correlated with enhanced ALDH1A1 expression and Aldefluor activity (Dylla et al. 2008). In addition to the link between ALDH activity and cyclophosphamide resistance, there is also a general association of ALDH activity/Aldefluor positivity with resistance to other chemotherapeutics. Breast tumor samples with high levels of ALDH1A1 associated with patient resistance to paclitaxel and epirubicin (Tanei et al. 2009). Aldefluor$^+$ isolated Ewing's sarcoma cells, from human cell lines and patient-derived xenografts had CSC properties and were resistant to doxorubicin (Awad et al. 2010). Aldefluor$^+$ subpopulations from lung cancer cells had increased resistance to multiple chemotherapeutic agents (cisplatin, gemcitabine, vinorelbine, docetaxel, doxorubicin and daunorubicin) and lung cancer patients with high ALDH1A1 had worse outcomes (Jiang et al. 2009). ALDH1A1 expression confers gemcitabine resistance to pancreatic cancer cells (Duong et al. 2012). Gastric CSCs with high ALDH activity exhibited increased resistance to 5-fluorouracil and cisplatin (Nishikawa et al. 2013). Similarly, ALDH activity attributed to ALDH1A1 protected a chemoresistant population of gastric cancer cells by reducing reactive oxygen species and consequently DNA damage and apoptosis (Raha et al. 2014). There is also clinical evidence of ALDH imparting chemoprotectant properties. Patients with locally advanced breast cancer were treated with docetaxel and FEC 100 (an anthracycline-based drug); of the patients who did not have a complete response, if the remaining tumor cells had high ALDH1 expression this was strongly predictive of worse overall survival (Alamgeer et al. 2014).

Unlike the well-described mechanism with cyclophosphamide, it is unclear whether these other drugs are metabolized directly by ALDH enzymes or if ALDHs minimize their cellular toxicity by clearing reactive aldehydes generated during

their primary mode of action. Alternatively, it is also possible that ALDH activity confers resistance by influencing cell signaling cascades such as the embryonic cell signaling pathways Notch and Hedgehog (Nishikawa et al. 2013). This is possible considering that three of the ALDH isoforms ALDH1A1, ALDH1A2 and ALDH1A3 are critical in the retinoic acid (RA) signaling pathway, and that two of these, ALDH1A1 and ALDH1A3 are expressed in the CSC populations of multiple cancers. The ALDH1A enzymes are the only enzymes that can generate RA from retinal (Coyle et al. 2013; Marcato et al. 2011a). Through their role in RA production, the ALDH1A enzymes can regulate expression of up to thousands of genes, influencing cell death, proliferation, and differentiation. Cross-talk between the RA cell signaling pathway and the embryonic cell signaling pathways is common. Therefore, evidence of ALDH-mediated chemoresistance related to Notch or Hedgehog signaling may be connected to ALDH1A-mediated RA signaling.

3.6 Chemoresistance of Cancer Stem Cells Due to Other Detoxification Mechanisms

ALDHs are not alone in detoxifying drugs and imparting chemoresistance to cancer cells; other proteins, in particular the cytochrome P450 oxidase (CYP) super family of enzymes metabolize endogenous molecules and xenobiotics (Gillet and Gottesman 2010). Some CYPs are highly expressed in cancer and have been shown to have an active role in both the activation of prodrugs (Kiyotani et al. 2012) and chemoresistance (Cizkova et al. 2012; Ripert et al. 2013; Xu et al. 2012). Human express 57 CYPs in 18 families and members of the CYP1, CYP2, and CYP3 families are particularly important in the detoxification of chemotherapeutics and other xenobiotics. For example, CYP2C and CYP3A contribute to the metabolism of taxanes (docetaxel and paclitaxel) (Cresteil et al. 1994; Gustafson et al. 2005; Royer et al. 1996). There is some evidence of increased CYP expression in CSCs, associated with chemotherapy resistance. Putative colon CSCs had increased CYP3A expression (Olszewski et al. 2011). Hypoxia-induced CYP2C9 imparted doxorubicin resistance to SP-identified liver CSCs (Myung et al. 2012). Future studies will likely reveal further association between CSCs, CYP enzymes and chemoresistance.

4 Chemoresistance of Cancer Stem Cells by Enhanced DNA Repair Mechanisms

4.1 Enhanced DNA Repair in Stem Cells

High fidelity and genomic stability is required in normal stem cells to maintain multipotency and extensive cycles of self-renewal. This tight control of DNA fidelity is essential to prevent damage from being amplified in further generations. Evidence

is accumulating that adult stem cells mediate this capability by enhancing the DNA damage response. Several studies have found higher expression of DNA repair genes (e.g. ERCC2, ERCC5, Ku80, MSH2, RAD23B) in murine stem cell populations (Ivanova et al. 2002; Ramalho-Santos et al. 2002). Other studies have reported differing response rates to DNA damage when stem cell populations are compared to progenitor cells (Maynard et al. 2008; Mohrin et al. 2010; Rossi et al. 2007; Sotiropoulou et al. 2010).

While the evidence remains murky about the origin of CSCs, there is little doubt that they rely on many of the same characteristics as adult stem cells – enhanced self-renewal and the ability to differentiate into a heterogeneous population of daughter cells. It is thus likely that the enhanced DNA repair observed in normal stem cells would be observed in CSCs – and possibly confers resistance to DNA-damaging agents.

4.2 DNA-Damaging Agents

Several of the first anticancer agents, nitrogen mustards and folate antagonists, were identified as DNA-damaging agents (Farber 1949; Gilman 1963; Gilman and Philips 1946; Mattes et al. 1986; Povirk and Shuker 1994). The often-impaired DNA repair mechanisms associated with many cancer cells makes them exquisitely sensitive to these drugs. Furthermore, the rapid proliferation of cancer cells requires relaxed DNA damage-sensing and repair capabilities, which makes them more sensitive to DNA damage. This sensitivity has been exploited by DNA-alkylating agents cyclophosphamide, chlorambucil, and mephalan, which are all derivatives of nitrogen mustards. In addition, the accidental discovery of cisplatin as an anticancer agent inspired the generation of platinum-based analogs such as carboplatin and oxaliplatin (Rosenberg et al. 1965; Wheate et al. 2010). These platinum agents form adducts primarily on guanine bases. When these adducts form on adjacent bases, intrastrand crosslinks are generated (Kelland 2007; Siddik 2003).

4.3 Mechanisms of DNA Repair

Both the nitrogen mustards (alkylating agents) and platinum agents can cause double-strand breaks (DSBs) in DNA (Fig. 17.1), which need to be repaired if cells are to survive. Repair of DSBs is typically done through the homology-directed repair pathways or nonhomologous pathways (Mehta and Haber 2014). Homologous recombination (HR) is a relatively accurate repair process as the undamaged sister chromatid can be used as a template; however, it may result in chromosomal crossover. HR is thus most effective during late S and G2 phases of the cell cycle. Following a DSB, HR is initiated once 3' single-stranded DNA overhangs are generated. The RAD51 recombinase is recruited to the overhangs and generates a

nucleoprotein complex. This complex can invade the sister chromatid at the identical sequence. In contrast, non-homologous end-joining (NHEJ) is most likely activated during G1 or early S phase when a sister chromatid cannot be found. During NHEJ, the Ku70/Ku80 heterodimer binds the broken ends of the DNA. Once Ku proteins are bound, NHEJ directly ligates the two strands. This process is error-prone and frequently results in insertions, deletions or substitutions at the break site.

For the most part, mammalian cells resort to p53-dependent apoptosis instead of developing an excess capacity for DNA repair when faced with irreparable damage. However, approximately 50% of all human cancers have somatic mutations in the p53 gene, often inactivating this response (Soussi et al. 2006). Thus, some cancers exhibit enhanced DNA repair as a mechanism of resistance to DNA damaging chemotherapeutic agents, whether it is intrinsic or acquired throughout the course of therapy (Parker et al. 1991; Zeng-Rong et al. 1995).

4.4 Evidence for Enhanced DNA Repair in Cancer Stem Cells

An important mechanism of resistance to DNA-damaging agents may be enhanced DNA repair (Fig. 17.3). Cells deficient in elements of DNA repair are more sensitive to chemotherapeutic-induced double-strand breaks (Lees-Miller et al. 1995; Ouyang et al. 1997). As discussed in detail previously in this chapter, drug efflux is a major contributing factor to the chemoresistance of CSCs. Evidence from glioblastoma suggests that CSCs are chemoresistant independent of drug efflux, suggesting that other mechanisms, such as enhanced DNA repair, also contribute to chemoresistance of these cells (Eramo et al. 2006). The DNA damage response in glioblastoma CSCs has been the most thoroughly investigated. Glioblastoma is often treated with temozolomide, an alkylating agent. Temozolomide alkylates guanine residues to O6-methylguanine (O6-MeG) (Zhang et al. 2012). This can be repaired by O-6-methylguanine-DNA methyltransferase (MGMT). If the lesion is not repaired, O6-MeG pairs with thymine during DNA replication and DNA mismatch repair machinery excises the mispaired thymine (Casorelli et al. 2008). The DNA lesion persists on the template strand, causing repeated (and futile) cycles of mismatch repair. This ultimately results in collapse of the replication fork, triggering cell cycle arrest and apoptosis. Thus, low levels of MGMT and functional mismatch-repair machinery are essential for the response of these cells to temozolomide. In $CD133^+$ glioma CSCs, higher levels of MGMT have been detected, conferring increased resistance to temozolomide therapy (Liu et al. 2006; Pistollato et al. 2010). However, controversial data has been presented where temozolomide selectively targets the CSC population of glioblastoma (Beier et al. 2008). Further evidence will be required to definitively determine the contribution of CSCs to the chemoresistance of glioblastoma (Pallini et al. 2009; Persano et al. 2012).

Enhanced DNA repair has been reported in CSCs of other cancer types. In a model of p53-null breast cancer, the CSC population had higher expression of DNA damage response and repair genes (e.g. BRCA1, UNG, XRCC5) (Zhang et al.

2008a). An MCF7 CSC-identified population demonstrated a more active single-strand break repair pathway (Karimi-Busheri et al. 2010). Prostate CSCs have been observed to have increased copy numbers of BRCA1 and RAD51 (Mathews et al. 2011). In neurospheres derived from pediatric brain tumor cells, DNA was repaired more quickly (Hussein et al. 2011).

Alternatively, other mechanisms may confer protective advantages to CSC DNA. In fact, evidence has suggested that leukemic CSCs may have a lower capacity for DNA repair, providing support for the existence of an alternate hypothesis (Buschfort-Papewalis et al. 2002). Constitutive activation of the cell cycle checkpoint response would provide CSCs more time to repair DNA lesions before replication (Ropolo et al. 2009; Viale et al. 2009; Zhang et al. 2008a). Likewise, quiescence in CSCs would minimize damage to the CSC genome, and evidence for resistance to DNA-damaging agents by quiescence is discussed in more detail in the next section.

5 Chemoresistance of Cancer Stem Cells by Quiescence

A cell in prolonged G0 phase of the cell cycle is quiescent. One hallmark of adult stem cells is their relative quiescence (Orford and Scadden 2008). Some evidence suggests that the CSCs of at least some cancers may have this property; this would impart them with intrinsic resistance to many chemotherapeutics (Guzman and Jordan 2009). Quiescence, or slower progression through the cell cycle, would render these cells less susceptible to chemotherapeutics. If a subpopulation of tumor cells is slower-dividing or quiescent, they would be relatively resistant to these anticancer drugs, especially to antimitotics and DNA-damaging drugs which require cell division for efficacy. However, the evidence is inconclusive regarding this potential characteristic of CSCs.

The gene expression profiles of $CD133^+$-identified glioblastoma CSCs from treatment-refractory recurrent tumors were consistent with quiescent cells (Liu et al. 2009). Similarly, Pece et al. determined that high-grade breast tumors enriched in CSCs had gene expression profiles consistent with quiescent mammary stem cells (Pece et al. 2010). Furthermore, in comparison to $CD133^-$ cancer cells, $CD133^+$-identified CSCs isolated from hepatocellular carcinoma were more resistant to doxorubicin and 5-fluorouracil, potentially, in part due to activation of pro-quiescence pathways (Ma et al. 2008b). In leukemia, quiescent tumor initiating cells are highly resistance to chemotherapy and play an important role in promoting disease relapse (Wang and Dick 2005). For example, quiescent CML CSCs were resistant to both imatinib and dasatinib tyrosine kinase inhibitors (Copland et al. 2006). In ovarian cancer, putative CSCs identified by $CD24^+$ expression were quiescent and were resistant to cisplatin compared to the $CD24^-$ cancer cells (Gao et al. 2010). It should be noted that the $CD24^+$ ovarian CSCs were enriched for cells in the S phase of the cell cycle compared to the $CD24^-$ population. While this would

typically confer a higher proliferation rate in the cells, the authors noted that it was insufficient for the $CD24^{+}$ cells to finish progression through the cell cycle.

In normal stem cells, several intrinsic and extrinsic mechanisms regulate quiescence, including tumor suppressor p53, mammalian target of rapamycin (mTOR), and hypoxia inducible factor 1 α (HIF1α) (Li and Bhatia 2011). Interestingly, multiple studies have linked these quiescence inducing factors to CSCs and their resistance to chemotherapy. In a $BRCA1/p53^{-}$ spontaneous mouse mammary tumor model, $CD29^{high}CD24^{medium}$-identified CSCs contribute to cisplatin resistance of the tumors (Shafee et al. 2008). In a SP of MCF7 cells with CSC tumorigenic properties and increased expression of MDR genes MDR1/ABCB1 and BCRP/ABCG2 and altered mTOR signaling, gene expression and cell cycle analysis suggested they were quiescent (Zhou et al. 2007). HIF1α overexpression has been associated with tumor progression and metastasis in different types of tumors including, lung, breast, and ovarian cancers (Zhong et al. 1999). Additionally, HIF1α regulates drug efflux transporter MDR1/ABCB1 (Comerford et al. 2002). Furthermore, hypoxia inducible factors have been associated with tumorigenicity regulation in glioma CSCs (Li et al. 2009). Together, these studies suggest an important role for HIF1α in regulating chemotherapy resistance in quiescent CSCs.

One approach to CSC-targeted therapy may be inducing cell cycle entry or promoting cell cycle progression. A 2010 study indicates that stimulating quiescent leukemia cells to divide can improve the efficacy of some chemotherapies, such as those which are cell cycle dependent (Saito et al. 2010). Additional investigation into the effect of disturbing quiescence in CSCs by targeting its various regulators may provide a breakthrough in enhancing chemotherapy efficacy and patient survival.

6 Cancer Stem Cell Chemoresistance Due to Apoptosis Inactivation

An important response of cancer cells to chemotherapeutics that target rapidly-dividing cancer cells (such as paclitaxel and doxorubicin) is activation of apoptosis. Cancer cells demonstrate several mechanisms which interfere with drug-induced apoptosis including manipulation of the B-cell CLL/lymphoma 2 (BCL-2) protein family. Under stress conditions, the cell fate decision to undergo apoptosis is governed by the interaction between different components of the BCL-2 family. The stress signal is carried by BAD and BIM, which interact with pro-survival BCL proteins (e.g. BCL-2, BCL-xL) and inhibit their repression of pro-apoptotic proteins BAX and BAK; which in turn activate apoptotic pathways and commit the cell to apoptosis (Adams and Cory 2007).

The role of the BCL-2 family in tumorigenesis has been heavily investigated since the early 1990s. Interestingly, p53 was found to regulate apoptosis in a BCL-2-dependent manner. Decreased levels of p53 resulted in an increase in BCL-2 levels and a decrease in BAX levels in murine leukemia cells (Miyashita et al. 1994). These observations indicate the important role for p53 loss in promoting BCL-2

mediated resistance to chemotherapy induced apoptosis (Fig. 17.3). This finding was confirmed in human malignancies as p53 was found to be a direct transcription activator of Bax gene. BCL-2 inhibition of apoptosis was found to confer resistance to several chemotherapeutics. Overexpressing BCL-2 in breast cancer MCF7 cells increased their resistance to doxorubicin treatment (Davis et al. 2003). Furthermore, inhibition of interleukin 10 (IL10) and subsequent down-regulation of BCL-2 by rituximab enhanced the sensitivity of non-Hodgkin's lymphoma to chemotherapy (Alas and Bonavida 2001).

Given the aggressive nature of CSCs and their high resistance to chemotherapy, several studies investigated the role of BCL-2 in mediating this resistance. Interestingly, overexpression of BCL-2 in myeloid progenitor cells promoted leukemia development in trangenic mice suggesting a potential overlap between BCL-2 expression and the ability of the cancer initiating cells to form tumors (Jaiswal et al. 2003). This overlap was also observed in glioblastoma, as $CD133^{+}$-identified CSCs had greater BCL-2 expression and increased resistance to chemotherapeutics (Liu et al. 2006; Shervington and Lu 2008). Additionally, $CD133^{+}$ hepatocellular carcinoma CSCs were resistant to doxorubicin and 5-fluorouracil and overexpressed BCL-2 (Ma et al. 2008b). Identifying BCL-2 as a mediator of CSC chemotherapy resistance has raised the possibility of developing novel methods of targeting MDR in CSCs. Several BCL-2 targeting drugs, including oblimersen sodium (Genasense), are being investigated to determine their effect in combination therapy (Kang and Reynolds 2009; Ma et al. 2008b).

7 Role of Cancer Stem Cell-Related Signaling Pathways in Chemoresistance

Many aggressive cancers are associated with MDR, making effective cytotoxic therapy a difficult challenge. Embryonic signaling pathways such as wingless-related (Wnt), Notch, and Hedgehog have been implicated in this resistance in numerous cancer types. We discuss below the evidence for the involvement of Wnt, Notch, and Hedgehog in chemoresistance, specifically focusing on examples related to CSCs (Fig. 17.3). For an overview of these pathways, their signaling components, evidence for their increased activation in CSCs, and in-depth discussion of potential targeting strategies, please see Chap. 15.

7.1 Wnt Signaling in Chemoresistance

A well-studied paradigm of Wnt signaling-mediated chemoresistance in cancer is neuroblastoma. Neuroblastoma is an aggressive childhood cancer. The origin of neuroblastoma from primitive cells in the sympathetic nervous system leads to the characterization of neuroblastoma as a developmental disease. The Wnt signaling

pathway has been described as aberrantly activated in neuroblastoma cells lacking MYCN amplification (Liu et al. 2008). Investigations of chemoresistance in neuroblastoma have indicated Wnt signaling as a contributing factor. Doxorubicin-resistant cells have higher expression of Wnt receptor Frizzled 1 (FZD1); silencing of FZD1 decreased expression of ABC transporter MDR1/ABCB1 and restored doxorubicin sensitivity. This in vitro work correlated with samples of neuroblastoma tumors pre- and post-chemotherapy (Flahaut et al. 2009), indicating that a complex pathway between Wnt and MDR1 may regulate resistance to doxorubicin (Fig. 17.3). Interestingly, $CD133^+$-identified neuroblastoma CSCs have been demonstrated to be more resistant to doxorubicin; however, Wnt-inhibition sensitized these cells to doxorubicin treatment (Vangipuram et al. 2012). This suggests that Wnt activation may be essential for the chemoresistance of $CD133^+$ neuroblastoma cells.

Pancreatic cancer is often characterized by its resistance to many cytotoxic agents used in cancer therapy. There is evidence to suggest that Wnt signaling plays a role in the resistance of pancreatic cancer cells to trichostatin A (a histone deacetylase inhibitor) which has been tested pre-clinically with other chemotherapeutic agents (Wang et al. 2014a). Similarly, a 2013 study identified that gemcitabine resistance may be mediated by Wnt signaling (Nagano et al. 2013).

Ovarian cancer is another highly aggressive cancer with poor survival rates. Ovarian CSCs demonstrate resistance to cisplatin/paclitaxel which is c-Kit and Wnt dependent (Chau et al. 2013). Silencing of Wnt2B or β-catenin enhances sensitivity of ovarian cancer cells to cisplatin or paclitaxel (Wang et al. 2012; Zhao et al. 2014). In patients with advanced ovarian cancer, strong membranous β-catenin expression was associated with resistance to platinum agents (Bodnar et al. 2014), providing support for the involvement of Wnt signaling in chemoresistance of ovarian cancer.

In hepatocellular carcinoma, putative $OV6^+$ CSCs display highly active Wnt signaling and are more resistant to standard chemotherapy. Inhibition of Wnt signaling or knockdown of β-catenin expression decreases the frequency of these CSCs and chemoresistance is reversed (Yang et al. 2008). Wnt signaling has been implicated in the resistance of some hepatocellular carcinoma patients to interferon-α/5-fluorouracil treatment. Activating Wnt signaling via treatment with a GSK3β inhibitor induced chemoresistance of hepatocellular carcinoma cells in vitro (Noda et al. 2009). These studies support the targeting of Wnt signaling for induction of chemosensitivity in hepatocellular carcinoma.

Wnt signaling is important for the self-renewal of hematopoietic stem cells (HSCs). When Wnt signaling is inhibited, inhibition of HSC growth in vitro and reduced reconstitution in vivo is observed (Reya et al. 2003). This may explain the role of Wnt signaling in the development of BCR-ABL CML (Zhao et al. 2007).

There is evidence to suggest Wnt mediates chemoresistance in other cancer types. Treatment of prostate cancer cells with Wnt inhibitors reduced protasphere size and self-renewal (Bisson and Prowse 2009). As measures of tumorigenicity, this indicates modulation of the CSC characteristics via Wnt signaling. Overexpression of secreted frizzled related protein 1 (SFRP1) reverses the chemoresistance of

taxane-resistant lung adenocarcinoma cells (Ren et al. 2014). Wnt signaling may also be involved in resistance to targeted therapy such as BRAF inhibitors. About half of melanoma tumors can be treated with BRAF inhibitors; however, resistance can occur via a variety of mechanisms. A 2014 study identified an increase in WNT5a in BRAF-inhibitor-resistant melanoma tumors, and RNAi-mediated reduction of WNT5a increased sensitivity (Anastas et al. 2014).

7.2 Hedgehog Signaling in Chemoresistance

Evidence suggests that inhibiting Hedgehog signaling increases the response of cancer cells to multiple unrelated chemotherapies. Hedgehog contributes to chemoresistance by increasing drug efflux via ABC transporters. Hedgehog has been found to regulate MDR1/ABCB1 and BCRP/ABCG2 (Fig. 17.3). In diffuse large B-cell lymphoma, Singh et al. demonstrated that ABCG2 is a direct target of Hedgehog signaling via a GLI-binding site in the ABCG2 promoter (Singh et al. 2011). Inhibiting expression of ABCB1 or ABCG2 partially reversed Hedgehog-mediated chemoresistance (Sims-Mourtada et al. 2007); while activating Hedgehog signaling increased drug resistance (Singh et al. 2011).

In addition to the contributions of Wnt signaling, Hedgehog signaling also mediates the multidrug resistance of pancreatic cancer. Pancreatic cancer tumorspheres display high levels of Hedgehog components, implicating Hedgehog in CSC self-renewal and differentiation. These tumorspheres also display resistance to gemcitabine; however, when treated with the Hedgehog inhibitor cyclopamine, this resistance was reversed (Huang et al. 2012; Yao et al. 2011). The role of Hedgehog signaling in chemoresistance may be due to several effects. First, ABC transporters are often overexpressed in pancreatic cancers, which may be regulated by Hedgehog-Gli signaling (Santisteban 2010). It may also be due to hypoperfusion of pancreatic tumors, which results in decreased drug delivery. In a mouse model of pancreatic ductal adenocarcinoma (PDAC), treatment with a Hedgehog inhibitor transiently increased intratumoral vascularisation and aided the delivery of gemcitabine (Olive et al. 2009).

Overexpressing Hedgehog pathway components induces chemoprotection of myeloid leukemic cells; whereas inhibition confers drug sensitivity (Queiroz et al. 2010). This implicates the Hedgehog pathway as essential for chemoresistance in myeloid leukemia (Liu et al. 2014b). Hedgehog signaling is also implicated in the chemoresistance of CSCs and other cancer types. These include paclitaxel-resistant breast cancer cells and putative prostate CSCs (Chai et al. 2013; Singh et al. 2012); cisplatin-resistant ovarian CSCs (Steg et al. 2012); and temozolomide-resistant $CD133^{+}$-identified glioma CSCs (Ulasov et al. 2011). Glioma CSCs often demonstrate activated Notch and Hedgehog signaling, which is enhanced upon temozolomide treatment. Importantly, inhibition of these pathways with a γ-secretase inhibitor or cyclopamine modulated the resistance of glioma CSCs to temozolomide (Ulasov et al. 2011).

7.3 Notch Signaling in Chemoresistance

Notch signaling has been implicated in the resistance of many cancers to various unrelated cytotoxic drugs. For example, the Notch1 receptor is highly expressed in cisplatin-resistant cells of head and neck squamous cell cancers, colorectal cancers, and ovarian carcinomas (Aleksic and Feller 2008; Gu et al. 2010; Zhang et al. 2008b, 2009). In colon cancer, treatment with oxaliplatin, 5-fluorouracil, or SN-38 (the active metabolite of irinotecan) induces the Notch intracellular domain (NICD) (Meng et al. 2009). Inhibiting Notch signaling with a γ-secretase inhibitor sensitized these cells to chemotherapy, implicating Notch signaling via NICD in chemoresistance. Interestingly, other Notch-dependent mechanisms have been indicated in the chemoresistance of pancreatic cancer (Du et al. 2013, 2014; Kang et al. 2013). Inhibition of Notch signaling is also able to counteract the chemoresistance of other cancer types including gliomas (Ulasov et al. 2011), osteosarcoma (Ma et al. 2013), and multiple myeloma (Xu et al. 2012).

There is also evidence of Notch-related chemoresistance specific to CSCs. For example, ovarian CSCs expressing increased Notch1 are more resistant to cisplatin and paclitaxel than their non-CSC counterparts (Zhang et al. 2008b). Furthermore, Notch3 overexpression increases platinum chemoresistance, while treatment with γ-secretase inhibitor depletes CSC frequency and increases platinum sensitivity of ovarian cancer cells (McAuliffe et al. 2012). In lung adenocarcinoma, cisplatin selects for $CD133^{+}$ putative CSCs and Notch-mediated multidrug resistance (Liu et al. 2013). In pancreatic cancer, Notch2 and ligand JAG1 are up-regulated in gemcitabine-resistant cancer cells; siRNA inhibition of Notch signaling partially reversed the epithelial mesenchymal transition phenotype associated with CSC-like cells and increased their sensitivity (Wang et al. 2009). Notch-mediated epithelial to mesenchymal transition has been implicated in chemoresistance in other cancer types as well, including erlotinib resistance in lung cancer, oxaliplatin resistance in colorectal cancer, and paclitaxel resistance in ovarian cancer (Kajiyama et al. 2007; Sabbah et al. 2008; Yang et al. 2006; Yauch et al. 2005). Finally, Notch signaling is also connected ABC transporter chemoresistance mechanisms of CSCs as Notch1 transactivated ABCC1 expression, which led to increased docetaxel resistance in prostate CSCs (Liu et al. 2014a).

8 Overcoming Chemoresistance by Targeting Cancer Stem Cells

Conventional therapies have been mostly successful in reducing tumor bulk; however, recurrence is still a major concern for many cancer types. For example for, breast cancer, in approximately a quarter of treated patients eventually recur with metastatic disease and succumb to the disease. It is hypothesized that targeting CSCs will reduce cancer recurrence and thus mortality (Fig. 17.2). Based on some

of the chemoresistance mechanisms discussed above (Fig. 17.3), there are many potential avenues for overcoming CSC chemoresistance and effectively targeting these highly tumorigenic populations.

As discussed in detail in Chap. 15 and mentioned earlier, the embryonic signaling pathways, Wnt, Notch, and Hedgehog, are dysregulated in CSCs; with CSCs becoming dependent on these pathways. This presents many potential therapeutic targets that are being heavily exploited and those strategies are discussed in detail in Chap. 15. Herein, we will review other mechanisms of selectively targeting CSCs, such as ALDH inhibitors, ABC transport protein inhibitors, and epigenetic modulators.

8.1 Aldehyde Dehydrogenase Inhibitors

As discussed earlier, increased ALDH activity is a common biomarker of CSCs and is involved in detoxifying certain chemotherapeutics. Furthermore, recent evidence suggests that ALDHs, in particular the ALDH1A3 isoform via inducing RA signaling, are active determinants of cancer progression (Luo et al. 2012; Mao et al. 2013; Marcato et al. 2014; van den Hoogen et al. 2010). These multiple roles in cancer make targeting ALDHs with specific inhibitors a promising avenue for novel anticancer and CSC therapy development. Known inhibitors of ALDHs include disulfiram, ampal, benomyl, citral, chloral hydrate, chlorpropamide analogues, coprine, cyanamide, CVT-10216, benzimidazole-based analogues, daidzin, DEAB, gossypol, kynurenine tryptophan metabolites, molinate, pargyline, and nitroglycerin (Fig. 17.4) (Koppaka et al. 2012; Pors and Moreb 2014). The inhibition of specific isoforms is unknown for most of these inhibitors; however, some have reported differential inhibitory concentrations for ALDH1A1, ALDH3A1 and ALDH2 as reported in recent reviews (Koppaka et al. 2012; Pors and Moreb 2014).

Of these inhibitors, disulfiram is the most extensively studied and has generated the most recent interest with regards to potentially treating cancer and targeting CSCs. Disulfiram has been used in the clinic for decades to treat alcohol abuse. Also known as Antabuse or Antabus, disulfiram inhibits the breakdown of alcohol metabolite acetaldehyde by liver enzymes ALDH1A1 and ALDH2 (which are also known as acetaldehyde dehydrogenases) to acetic acid. The accumulation of acetaldehyde by disulfiram causes nausea and other symptoms which deters alcohol consumption. In addition to its use in the treatment of alcoholism, there is increasing indication that disulfiram has anti-cancer activity, by potentially multiple mechanisms. For breast cancer, it was reported that disulfiram reduced tumor growth in MDA-MB-231 breast cancer tumor xenografts (Chen et al. 2006). However, the authors suggest that this anti-breast cancer activity is due to disulfiram complexing with copper (found endogenously in the tumor microenvironment), which results in proteasome inhibition (Chen et al. 2006). The authors did not assess if inhibition of ALDH enzymes was a factor in the anti-breast cancer activity of disulfiram. Copper-complexed disulfiram also inhibited the growth of non-small cell lung cancer cells

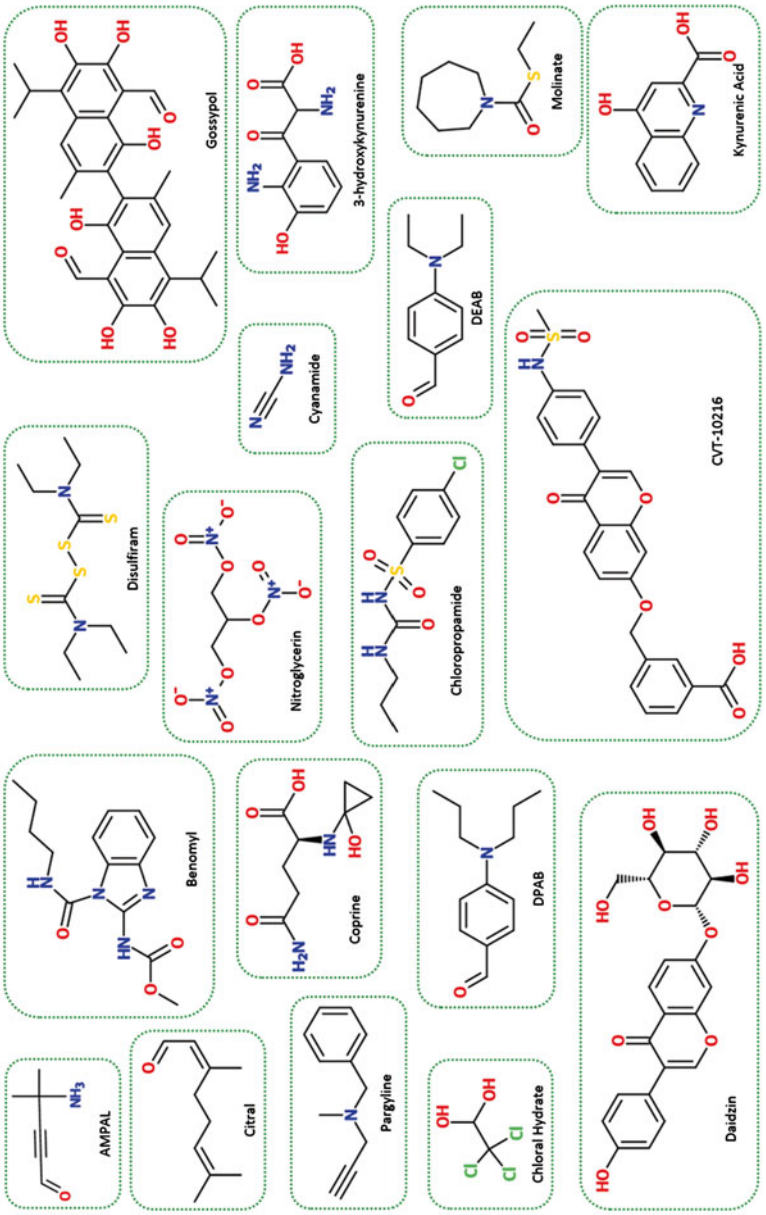

Fig. 17.4 Structures of known aldehyde dehydrogenase inhibitor molecules. ALDH inhibitors include AMPAL (4-amino-4-methyl-2-pentyne-1-al), benomyl (methyl1(butylcarbamoyl)-2 benzimidazole-carbamate), citral (3,7-dimethyl-2,6-octadienal), chloral hydrate (trichloroacetaldehyde hydrate), chloropropamide (1-(p-chlorobenzenesulfonyl)-3-propylurea), coprine (N5-1-hydroxycyclopropy l-L-glutamine), cyanamide, daidzin (4 h-1-benzopyran-4 one, 7-(beta-d glucopyranosyloxy)-3-(4-hydroxyphenyl)-5-hydro), CVT-10216, DEAB (diethylaminobenzaldehyde), DPAB (4-(N,N-dipropylamino)benzaldehyde), disulfiram (tetraethylthiuram disulfide), gossypol, kynurenic acid, 3-hydroxykynurenine, molinate (S-ethyl hexohydro-1H-azepine-1-carbothioate), nitroglycerin (1,2,3-propanetriol trinitrate), and pargyline (N-methyl-N-2-propynyl-;Benzyl-methyl-2-propinylamine)

and sensitized them to cisplatin (Duan et al. 2014). These authors noted that the combination disulfiram and copper treatment decreased expression of ALDH1 and other stemness markers in the cancer cells. Furthermore, a high-throughput screen study identified disulfiram as an inhibitor of glioblastoma CSCs, which was potentiated when complexed with copper (Hothi et al. 2012). Other studies have shown that copper is not necessary for disulfiram-chemosensitization and targeting of CSCs. For example, disulfiram was shown to sensitize breast tumor xenografts to radiation treatment and tumor regression and coincided with a reduction in ALDH activity and other stemness markers (Wang et al. 2014d). Similarly, disulfiram chemosensitized a chemoresistant population of ALDH1A1-expressing gastric cancer cells (Raha et al. 2014). Disulfiram also reduced a population of sphere-forming putative liver CSCs (Chiba et al. 2014).

The success of these studies has fuelled interest in the use of disulfiram clinically. Multiple clinical trials are being conducted to test the potential use of disulfiram as cancer therapy by either complexing with copper and inhibiting proteosome activity or inhibiting ALDH1A1/CSC populations and sensitizing them to radiation and chemotherapeutics. These include phase I and phase II trials that are on either advanced or newly diagnosed glioblastoma muliforme (NCT00571116, NCT01907165, NCT00742911, NCT00312819 and NCT01777919). Pending the success of disulfiram in these trials, the clinical testing of other ALDH inhibitors will likely follow.

8.2 ABC Transporter Inhibitors

The association of increased ABC transporter expression and CSCs of various cancers has both applications for identifying CSCs based on their increased efflux capacity and as a method for targeting them using ABC transporter inhibitors as adjuvant therapy. Regardless of the potential for targeting CSCs, using ABC transporter inhibitors as adjuvant therapies has the potential benefit of generally increasing the efficacy of chemotherapeutics, with the hope that it may allow lower dosages of chemotherapeutics.

Inhibitors of ABC transporters typically work by one of three ways: by specific proteins interaction, by interfering with cellular ATP levels required to power ABC transporters, or by influencing the cellular permeability to ions required for ABC transporter function (Bartosiewicz and Krasowska 2009). These mechanisms illustrate the non-specificity of many ABC transporter inhibitors. Verapamil, a calcium channel blocker, and cyclosporin A, an immunosuppressive drug, are early examples of ABCB1/MDR1 and ABCG2/BCRP inhibitors. These drugs have shown some efficacy in the treatment of many cancers, including breast cancer, AML, and non-small cell lung carcinoma (Belpomme et al. 2000; Millward et al. 1993; Sonneveld et al. 2001). However, the high toxicity associated with these drugs has limited their clinical applications in the treatment of cancer, but has spurred the

development of second-generation drugs with increased specificity and hopefully reduced side effects. These included second-generation cyclosporin A analogue, valspodar; however, it too had limited clinical efficacy. For example, in a phase III study testing the effect of valspodar combined with paclitaxel and carboplatin versus paclitaxel and carboplatin in patients with advanced metastatic epithelial ovarian cancer or primary peritoneal cancer, the inclusion of valspodar did not improve patient outcomes and valspodar-treated patients experienced greater treatment-related toxicity (Lhomme et al. 2008). Third-generation ABC inhibitors with increased specificity are being developed and tested; however, these are also not showing significant promise. For example zosuquidar, a highly specific and potent inhibitor of MDR1/ABCB1, failed to improve outcomes of newly diagnosed AML patients (Cripe et al. 2010).

While ABC inhibitors were originally positioned as broad-spectrum drugs that would increase the efficacy of chemotherapeutics on all cells within a tumor, perhaps their greater utility will be in re-sensitization of a sub-population of tumor cells with pre-existing intrinsic resistance (i.e. CSCs) (Shukla et al. 2011). As proof of concept examples, verapamil can be used in conjunction with classic chemotherapies to target chemoresistant CSCs. Putative pancreatic CSCs were sensitized to gemcitabine (Hong et al. 2009), and $CD34^{+}CD38^{-}$-identified AML CSCs were sensitized to daunorubicin upon verapamil treatment (Ho et al. 2008). Furthermore, verapamil sensitized $Aldefluor^{+}$-identified Ewing's sarcoma CSCs to doxorubicin (Awad et al. 2010).

Other drugs include first-generation (imatinib) and second generation tyrosine kinase inhibitors (e.g. nilotinib, dasatinib, tandutinib, and erlotinib), which were originally used in the treatment of CML with increased BCR-ABL activity (Dohse et al. 2010; Wu et al. 2014). These inhibitors have also shown anti-ABC transporter activity, with some showing efficacy that extends to CSC populations by multiple mechanisms, including affecting transporter expression levels, acting as transporter substrates (competitive inhibitors), and influencing their ATPase activity. For example, nilotinib significantly enhanced the cytotoxicity of doxorubicin and mitoxantrone in $CD34^{+}CD38^{-}$-identified leukemia CSCs cells and reversed MDR in primary leukemic blasts overexpressing ABCB1/MDR1 and ABCG2/BCRP (Wang et al. 2014b). Similarly, tandutinib reversed ABCG2/BCRP-mediated drug resistance in the SP of lung cancer A549 cells (Zhao et al. 2013). Erlotinib, also showed MDR1/ABCB1 and BCRP/ABCG2 inhibition activity by down-regulating their expression and modulating their ATPase activity. Furthermore, the ABC transporter inhibition activity of erlotinib extended to a subpopulation of putative AML CSCs in patient-derived samples (Lainey et al. 2012). The potential of ABC transport inhibitors to sensitize CSCs to other therapeutics remains a feasible option and the results of future clinical trials will determine the viability of this strategy.

8.3 *Epigenetic Modifiers to Resensitize CSCs to Chemotherapeutics*

Targeting epigenetic modifications to treat cancer is not a new idea (Kelly et al. 2010). Aberrant DNA methylation, histone modifications, and non-coding RNA expression, including microRNA (miRNA or miR) is intrinsic to cancer and the CSC population of many tumors often have distinct epigenetic changes (Gronbaek et al. 2007; Vincent and Van 2012; Babashah 2014; Babashah et al. 2012; Babashah and Soleimani 2011). This suggests that epigenetic-modifying drugs are a potential avenue for targeting CSCs (Fig. 17.5). It is unlikely that an epigenetic-based therapy will work alone; instead the aim is to transition the epigenome to a more chemosensitive state and use epigenetic therapies in tandem with conventional therapeutics.

DNA methylation is a key epigenetic mechanism in cancer which often silences tumor suppressor genes (TSGs) by adding methyl groups to CpG islands in the promoter regions of TSGs (Chen 2012; Dworkin et al. 2009; Gall and Frampton

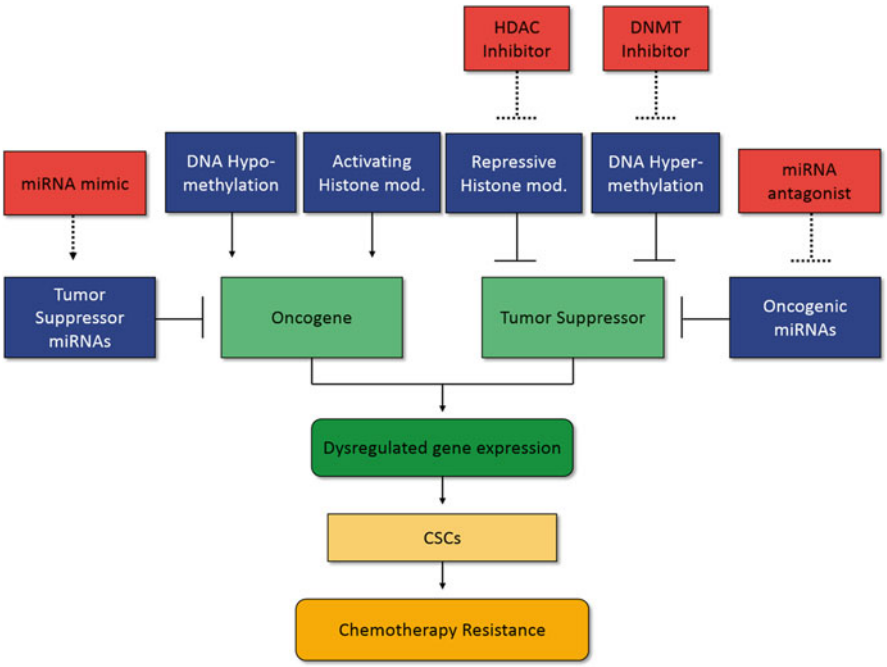

Fig. 17.5 Overcoming cancer stem cell chemotherapy resistance with epigenetic modifying drugs. CSCs have unique DNA methylation changes, histone modifications, and miRNA expression changes, which in addition to changing expression of tumor suppressor genes or oncogenes, also affect expression of many genes involved in chemoresistance. Therefore, by utilizing drugs that target these changes it also resensitizes CSCs to some chemotherapeutics

2013; Paul and Paul 2014; Suijkerbuijk et al. 2011; Van de Voorde et al. 2012). Most studies investigating the role of DNA methylation in chemoresistance do not distinguish CSCs from the tumor bulk and therefore a comprehensive methylation profile of CSCs is not available for discussion in the context of chemoresistance (Fujita et al. 2014; Klajic et al. 2014; Mo et al. 2015; Zhou et al. 2014b). Efforts to isolate these cells and then analyze the entire methylome would be welcome. There are several pathways described in the previous sections that could be under epigenetic control: for example inactivation of pro-apoptotic genes in CSCs (Sect. 6) may be due to silencing by hypermethylation. With this logic, DNA methyltransferase inhibitors (drugs that prevent the addition of methyl groups to DNA) are being investigated for the efficacy in resensitizing CSCs to chemotherapy.

Hepatocellular carcinoma, colorectal, glioblastoma, and ovarian CSCs are often characterized by CD133 positivity and the expression of CD133 in these cells is due to a loss of DNA methylation at the promoter region of CD133 (Baba et al. 2009; Yi et al. 2008). The demethylation of this gene can be partially attributed to exposure to TGF-β, which appears to inhibit DNA methyltransferase 1 (DNMT1) and DNMT3β (You et al. 2010). Inhibitors of TGF-β show potential in preventing the development of a chemotherapy resistant population of CSCs in in vivo breast cancer models (Bhola et al. 2013); this effect might be due to their DNMT inhibition, though it should be noted that TGF-β has other effects. In another breast cancer model, combination treatment of doxorubicin and a TGF-β inhibitor reduced the population of drug resistant CSC-like cells in vivo (Bandyopadhyay et al. 2010).

A recent study used a DNMT inhibitor (SGI-110) to treat ovarian cancer in vivo and showed that DNMT inhibition is able to resensitize ovarian CSCs to cisplatin treatment and delay recurrence (Wang et al. 2014c). Demethylation of thousands of CpG sites was observed, and it was proposed that the overall loss of the "epigenetic brakes" established by DNA methylation allowed the CSC population to differentiate and become sensitive to platinum therapy. This promising data supports the results of a phase II clinical trial showing that patients with heavily pre-treated or platinum-resistant ovarian cancer could become resensitized to carboplatin and experienced longer progression-free survival (Matei et al. 2012).

Several DNA methyltransferase inhibitors have been well characterized and are undergoing or have completed clinical trials for various malignancies (Connolly and Stearns 2012; Lyko and Brown 2005). 5-Azacytidine, 5-aza-2-deoxycytidine, and zebularine all act during the S-phase of the cell cycle and thus prefer rapidly dividing cells; however, as demonstrated in the ovarian cancer study mentioned above, these drugs may also be able to target CSCs.

Histone modifications are more plastic than DNA methylation and are controlled by the activities of histone-modifying enzymes that may add or remove modifications; the aberrant histone modifications seen in cancer arise from an imbalance of these enzymes (Esteller 2007; Plass et al. 2013; Yuen and Knoepfler 2013). Cancer cells often show dysregulation of histone methyltransferases and histone demethylases, and overexpression of histone deacetylases (HDACs). Like DNMT inhibitors, HDAC inhibitors are used to treat hematological cancers and are also in clinical trials for the treatment of solid tumors such as breast cancer (Connolly and Stearns 2012; Dhanak and Jackson 2014; Lyko and Brown 2005).

As discussed earlier, imatinib treatment has some efficacy in targeting CSCs; however, imatinib alone does not cure CML, potentially in part because the population of CSCs is not completely eliminated. With the addition of a HDAC inhibitor, quiescent CML progenitor cells were sensitized to imatinib treatment through a proposed mechanism of inhibiting genes essential to hematopoetic stem cell survival and maintenance (Zhang et al. 2010). Although a profile of CSC histone modifications is being developed, it is not yet clear how these histone changes are involved in chemoresistance.

Another potential approach to epigenetic therapies is manipulating miRNAs to inhibit oncogenes or promote expression of tumor suppressor genes in CSCs (Chhabra and Saini 2014). MiRNAs promiscuously target multiple genes and inhibit their expression; these short sequences regulate and are regulated by other epigenetic mechanisms. An advantage of targeting miRNAs is that the therapy will affect expression of multiple genes and pathways that are regulated by single miRNAs. The expression of miRNAs is dysregulated in cancer, especially in cells undergoing epithelial to mesenchymal transition, and there has been a recent interest in their use as therapies for targeting these invasive cells (Hu and Tang 2014). As CSCs are often associated with the epithelial to mesenchymal transition phenotype, miRNA expression manipulation may be used to effectively target the CSC population.

There are several "families" of miRNAs, one of the most prevalent in studies of cancer is the miR-200 family; this group of miRs is dysregulated in the CSCs of breast and lung cancers (Tellez et al. 2011). MiR-200 family expression is reduced by promoter hypermethylation and repressive histone modifications (H3K27me3) in $CD24^{-}CD44^{+}$-identified breast CSCs (Lim et al. 2013). ER-src-transformed cells injected into nude mice were treated with doxorubicin and tumor regressed; however regrowth of the tumors occurred post treatment cessation (Iliopoulos et al. 2010). However, when the mice received a combinatorial treatment of doxorubicin and miR-200b, relapse and tumor growth were almost entirely prevented. Analysis of the small tumors post-combination treatment showed a major reduction in the $CD24^{-}CD44^{+}$-identified CSCs (from ~15 % in tumors exposed to doxorubicin alone to <0.5 % in combination treatment). This study underscores the potential of miRNA modulation strategies as components of combination therapies for specifically targeting the CSC population.

MiRNAs are often dysregulated by aberrant DNA methylation in cancer. MiRNAs that inhibit oncogenes are often silenced by hypermethylation in tumors; this implies that DNMT inhibitors may be used to re-establish miRNAs control of certain oncogenes. Re-expression of miR-34a in human pancreatic CSCs and in human pancreatic cancer cell lines upon treatment with DNMT inhibitor 5-aza-2-deoxycytidine strongly inhibited cell proliferation, cell cycle progression, self-renewal, epithelial to mesenchymal transition and invasion in vitro (Nalls et al. 2011).

Therapies involving miRNAs may involve the use of miRNA antagonists or miRNA mimics depending on the gene targets of the miRs. For instance, application of miR-124 and miR-137 mimics to $CD133^{+}$ glioblastoma CSCs caused the cells the cells to differentiate in vitro (Silber et al. 2008). Therefore, miR-124 and/or miR-137 mimics are potential treatment options. MiRNA antagonists are also

showing some success in vitro; an antagonist of miRNA-21 was able to reduce cell proliferation and reduce the population of ALDH$^+$-identified CSCs in a breast cancer cell line model due to PTEN up-regulation and AKT/ERK1/2 inactivation (Han et al. 2012). Finally, with recent increasing evidence showing that other non-coding RNA, in particular long non-coding RNAs (lncRNAs), are playing an important role in cancer initiation, progression and drug resistance (Serviss et al. 2014; Yang et al. 2014; Zhou et al. 2014a), it remains a matter of time before the importance of lncRNA expression in CSC chemoresistance is also investigated.

9 Conclusions and Future Directions

Overwhelming increasing evidence suggests that CSC-associated chemoresistance is one of the most important hurdles to overcome in order to improve long-term cancer patient survival in the coming years. Many potential avenues for targeting CSCs are being explored and importantly, clinical trials are beginning to incorporate evaluation of CSC numbers before and after treatment, along with patient survival and tumor regression. These analyses will test the hypothesis supported by the cell culture and xenograft tumor regression model data suggesting that overcoming CSC-associated chemoresistance will significantly reduce cancer burden and improve overall treatment success. This will also have the desired side effect of increasing our understanding of CSCs and the role they play in cancer progression. Importantly, these studies will also reveal the cross-talk that is beginning to be evident between the diverse CSC-associated chemoresistance mechanisms.

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Chapter 18
Cancer Stem Cells and Tumor Radioresistance

Aadel A. Chaudhuri, Michael S. Binkley, and Maximilian Diehn

Abstract Cancer stem cells (CSCs) in a variety of tumor types have intrinsically greater resistance to ionizing radiation (IR) than the remaining cancer cells. Since surviving CSCs have the capacity to regenerate tumor deposits, CSC radioresistance represents an important clinical problem. Here we discuss mechanisms that CSCs employ to resist IR and therapeutic strategies that are currently being used in the clinic or are in various stages of development for overcoming these. While much ongoing work shows promise for increasing the efficacy of IR through rational targeting of CSCs, well-designed clinical trials testing such strategies will be required to bring these approaches into the clinic.

Keywords Cancer stem cells • Tumor radioresistance • Ionizing radiation • Surveillance

1 Introduction

In the last 50 years we have witnessed rapid advancements in cancer biology and oncology. Radiation therapy techniques have improved significantly, becoming more targeted, conformal and overall leading to improved survival and tumor control rates. Still, certain cancers such as glioblastoma are difficult to control locally and for nearly all cancers patients who develop disseminated disease remain largely incurable. Moreover, there are clinical examples of rapid tumor recurrence following treatment for nearly every type of cancer. Also post-treatment cancer

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surveillance remains mostly reliant on gross imaging techniques, without the ability to detect microscopic tumor foci. Enhanced understanding of cancer stem cells (CSCs) and the mechanisms they employ for resisting radiation therapy may thus help us in our quest for more effective therapies and better outcomes.

The primary therapeutic effect of ionizing radiation (IR) is the production of free radical reactive oxygen species (ROS) within cells, which in turn induce DNA damage. The most lethal type of DNA damage is double-strand breaks, since these have the highest probability of leading to cell death. The degree of radiation sensitivity of a tumor is affected by a variety of factors including cancer types, proliferation rate, degree of hypoxia of the tumor microenvironment, ability to repair DNA, and genetic mutations, to name a few. We and others have more recently shown that the presence of CSCs also contributes to radioresistance, both in vitro and in vivo (Diehn et al. 2009; Morrison et al. 2011; Bao et al. 2006).

Traditionally, assessment of treatment response in solid tumors has relied on tumor shrinkage as the primary clinical parameter, which may not be indicative of cancer stem cell (CSC) response since these often make up a minority of cells within a tumor. As CSCs often remain in quiescence, therapies acting on dividing cells may not be effective in targeting CSCs (Morrison et al. 2011). As one example, glioblastoma CSCs have demonstrated resistance to radiation therapy by increased cell cycle checkpoint signaling, allowing for repair of DNA damage during cell cycle arrest (Bao et al. 2006). In this chapter we will discuss mechanisms of CSC radioresistance, and strategies to overcome these.

2 Mechanisms of Radioresistance in Cancer Stem Cells

A number of studies have shown that normal and cancer stem/progenitor cells appear to harbor intrinsic resistance to IR compared to other cell types (Diehn et al. 2009; Morrison et al. 2011). For example, Woodward et al showed that normal progenitor cells in the mammary gland were more resistant to clinically relevant IR than nonprogenitors, and that overexpression of the Wnt/β-catenin pathway was capable of further enhancing radioresistance (Woodward et al. 2007). Examining CSCs, we found that in murine MMTV-*Wnt1* breast cancers this subpopulation displayed lower levels of DNA damage after IR than non-CSCs (Diehn et al. 2009). This translated to CSC enrichment in vivo in response to clinically relevant doses of IR. Furthermore, immunodeficient mice harboring head and neck cancer xenograft tumors experienced similar enrichment of the CSC population when treated with clinically relevant doses of IR (Diehn et al. 2009). Thus, both normal and malignant stem/progenitor cells appear to be intrinsically radioresistant.

CSCs have been shown to exploit a number of different mechanisms to exert resistance to IR (Fig. 18.1). One mechanism is by up-regulating expression of ROS scavenger pathway enzymes, the most important being Glutathione (GSH), a potent ROS scavenger (Diehn et al. 2009; Morrison et al. 2011; Phillips et al. 2006). For example, CSCs have been shown to up-regulate surface expression of the xCT

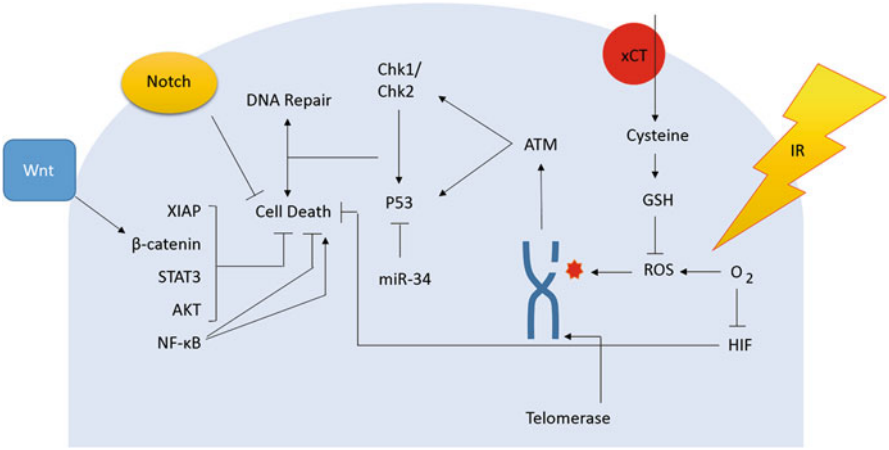

Fig. 18.1 *Mechanisms of tumor radioresistance*. Interconnecting pathways initiated by CSCs in response to ionizing radiation (IR) leading to radioresistance, involving Hypoxia Inducible Factors (HIF), Reactive Oxygen Species (ROS), Cysteine, Glutathione (GSH), Telomerase, ATM, Checkpoint kinases (Chk1/Chk2), p53, XIAP, β-catenin, STAT3, AKT, NFκB, Wnt and Notch. These pathways serve to make IR less effective at damaging DNA, improve chromosomal integrity, enhance DNA repair, and increase survival while inhibiting cell death

cysteine transporter, which is used to import cysteine and produce high levels of GSH (Ishimoto et al. 2011).

ROS scavenging cannot prevent all DSBs from forming and some CSCs are able to resist cell killing via enhanced DNA repair (Fig. 18.1). DSBs can induce reproductive cell death via the p53 pathway or can be repaired, in part mediated via activation of ATM and the checkpoint kinases Chk1 and Chk2 (Morrison et al. 2011). In some tumors, DNA repair in response to IR may be more robust in CSCs as a result of robust checkpoint signaling; increased checkpoint signaling induces more efficient cell cycle arrest, giving the cell more time to repair deleterious DNA damage. In a study of glioblastomas, radiation was shown to cause equal levels of DNA damage in both CSCs and non-CSCs, but CSCs were able to repair the damage more rapidly (Eyler and Rich 2008; Bao et al. 2006). Indeed, Bao et al. showed that the $CD133^{+}$ population in glioblastoma multiforme preferentially activated DNA damage checkpoints and exerted radioresistance by efficiently repairing IR-induced DNA damage.

In addition to augmented DNA repair systems and ROS scavenging mechanisms, CSCs may also exhibit enhanced telomerase function (Morrison et al. 2011) (Fig. 18.1). Telomerase, a complex ribonucleoprotein that synthesizes and maintains telomerase repeats at the end of chromosomes, is expressed highly in early embryonic development, and is subsequently turned off except for low level activity shown in some adult stem cell populations (Hiyama and Hiyama 2007). Enhanced telomerase function inhibits chromosomal degradation, which may contribute to resistance to IR (Rubio et al. 2004).

There are also some mechanisms extrinsic to CSCs that could contribute to their radioresistance. For example, solid tumors often outgrow their blood supply, leading to tumor hypoxia. Due to the decreased levels of oxygen, irradiation of hypoxic tumors leads to lower ROS production and less tumor cell killing (Kobayashi and Suda 2012). Intracellular hypoxia inducible factors (HIF) are up-regulated in this hypoxic environment, and may further promote CSC self-renewal and survival (Li et al. 2009).

3 Additional Putative Resistance Mechanisms to Ionizing Radiation and Other Treatments

CSCs have also been shown to have increased expression of pro-survival signaling pathways including elevated signaling via the XIAP, STAT3, Notch NFκB, AKT and Wnt/β-catenin signaling pathways (Fig. 18.1) (Morrison et al. 2011). In chronic myeloid leukemia (CML), the constitutively active BCR-ABL tyrosine kinase efficiently drives myeloid development leading to malignancy (Ren 2005). Imatinib has been shown to inhibit this critical kinase and halt tumor progression. However, CML CSCs are not addicted to this oncogenic signaling pathway, unlike their more mature counterparts; thus the disease usually recurs even when the disease is undetectable by RT-PCR after imatinib therapy is discontinued (Corbin et al. 2011). These examples serve to highlight that signaling pathways utilized by CSCs are complex, and may be unique from those utilized by more mature cancer cells from the same tumor type.

Over the last decade microRNAs (miRNAs, miRs) have proven to play increasingly important roles in cancer biology and stem cell biology (Babashah 2014). A number of miRNAs, including miR-125, miR-155, miR-146, miR-34, miR-17-92, let-7 and miR-21 have been shown to have oncogenic or tumor-suppressing roles (Calin and Croce 2006a, b; O'Connell et al. 2010b). Indeed, miR-125 has been shown to significantly modify and enhance hematopoietic stem and progenitor cells, allowing them to function as highly potent cancer stem cells capable of transmitting a serially transplantable aggressive leukemia in mice (O'Connell et al. 2010a; So et al. 2014; Chaudhuri et al. 2012). Similarly, up-regulating miR-155 or deleting miR-146a, miRNAs that are strongly induced by NFκB (Taganov et al. 2006; O'Connell et al. 2010b), alters the number and characteristics of hematopoietic stem and progenitor cells, leading eventually to myeloid malignancies (O'Connell et al. 2008, 2010a; Zhao et al. 2011). These miRNA-based mechanisms leading to malignancy have been shown to be via modulation of important signaling proteins including NFκB, AKT, p53, Ras and IRF4 (Chaudhuri et al. 2011, 2012; So et al. 2014; O'Connell et al. 2010b; Calin and Croce 2006a). Also, IR has been shown in vitro to alter expression of multiple miRNAs, an effect partially inhibited by pre-treatment with the ROS scavenger cysteine (Simone et al. 2009). Thus, modulation of key signaling pathways by miRNAs may enhance CSC survival and contribute to resistance to IR therapy.

4 Therapeutic Strategies for Overcoming Cancer Stem Cell Radioresistance

4.1 Microscopic Tumor Surveillance

A clinical challenge is not only definitive treatment of tumors harboring radioresistant CSC populations but also effective surveillance following treatment. We typically monitor patients following definitive IR treatment via frequent surveillance imaging with CT and/or PET-CT. While this is effective at evaluating gross tumor bulk, it is not an ideal method for predicting tumor recurrence, as it may miss detection of residual microscopic foci of radioresistant CSCs. For some solid tumors, blood-based biomarkers are available that partially address this problem. For example, in prostate cancer, post-treatment ultrasensitive PSA surveillance allows detection of biochemical recurrence even in the absence of grossly recurrent disease. In order to extend an analogous approach to a larger number of patients with different tumor types, we and others have recently developed highly sensitive methods for detecting circulating tumor DNA to enable microscopic disease surveillance (Newman et al. 2014; Kinde et al. 2013; Bettegowda et al. 2014). For example, we recently described an ultra-sensitive plasma surveillance method (called cancer personalized profiling by deep sequencing, CAPP-Seq) that detects cancer mutations in cell-free DNA isolated from blood to detect microscopic lung cancer recurrence earlier than possible by imaging surveillance (Newman et al. 2014) and are currently in the process of adapting the approach to other cancer types.

4.2 Targeting Checkpoint Signaling

Following exposure to IR, cell cycle arrest allows for repair of DNA damage and may represent a strategy for overcoming radiation resistance. In vitro analysis has demonstrated acquired radioresistance to conventional fractionation radiation, 2–3 Gy per fraction, by up-regulation of the cyclin D1/Cdk4 cell-signaling pathway (Shimura et al. 2011). Shimura et al. investigated the AKT and cyclin D1/Cdk4 pathways, which are expressed by tumor cells after irradiation and important in increasing the cell death threshold. Specifically, a Cdk4-inhibitor demonstrated synergy with fractionated IR in vitro, and increased apoptosis (Shimura et al. 2011).

Another alternative in overcoming radioresistance associated with conventionally fractionated radiation involves using higher doses of radiation per fraction administered. For example, doses of 10 Gy or higher have been observed to lead to cyclin D1 ubiquitin-proteosome degradation in tumor cells, as opposed to the up-regulation of the cyclin D1/Cdk4 noted above for lower doses, such as 2–3 Gy per fraction (Shimura et al. 2013). Higher doses of IR also lead to higher production of ROS and more double strand breaks, thus helping to overcome the free radical scavenging and DNA repair resistance mechanisms found in CSCs.

4.3 Hypofractionation of Radiotherapy and Overcoming Intrinsic Tumor Hypoxia

As an illustration of dose-dependent response, hypofractionated radiation techniques with higher dose per fraction using stereotactic techniques for early stage non-small cell lung cancer (NSCLC), also called stereotactic ablative radiotherapy (SABR), have proven highly effective with greater than 90 % local tumor control (Senan et al. 2011). Similarly, stereotactic radiosurgery (SRS) has proven effective for treatment of brain and spine metastatic lesions with very high rates of local control (Iwata et al. 2011). SABR and SRS likely exert their increased therapeutic benefit via a variety of mechanisms. First, higher doses will lead to more DSBs, thus increasing the chance that cancer cells will be unable to survive. Additionally, SABR/SRS may lead to stimulation of potent cytotoxic antitumor inflammatory responses (Yamada et al. 2008). There is disagreement on whether SABR/SRS primarily targets tumor cells or endothelial cells. Some investigators have suggested that the tumor endothelium is the primary target (Garcia-Barros et al. 2003) while more recent data suggests otherwise (Moding et al. 2014).

One theoretical disadvantage with hypofractionated radiotherapy is the limited oxygenation of hypoxic tumor cells that occurs between fractions. Conventional courses of radiation allow re-oxygenation of hypoxic tumor cells between fractions which aids in elimination of tumor cells. Tumor control probability modeling accounting for hypoxia has suggested the higher dose per fraction and non-homogeneous dose delivery used with SABR may compensate for the decreased period of oxygenation and shortened fractionation schedule (Ruggieri et al. 2010). Other studies have found decreased tumor cell killing using hypofractionated courses compared with conventional fractionation schedules by three orders of magnitude, attributing decreased cell kill to hypoxia and increased double-stranded DNA base repair under hypoxic conditions when using hypofractionated regimens (Carlson et al. 2011).

4.4 Making Ionizing Radiation More Potent with Radiosensitizing Agents

An attractive and intuitive method of making IR more effective at killing any cell type, including CSCs, is via concomitant administration of a radiosensitizing agent. The idea is to make a given dose of IR administered more tumoricidal than if IR were to be given alone. Clinically this approach is commonly used via concurrent administration of chemotherapy such as platinum-based agents in a variety of tumor types. While this approach has been shown to improve efficacy of IR, it also puts normal tissue within the IR field at greater risk for damage and usually leads to higher levels of toxicity. More targeted anti-CSC radiosensitizing approaches would therefore be desirable. Potential approaches include use of c-kit inhibitors in lung cancer, CD133 inhibitors in glioblastoma, and inhibition of free radical scavengers

(Levina et al. 2010; Fan et al. 2010; Biswas et al. 2005; Diehn et al. 2009). These agents offer a theoretical advantage over traditional chemotherapy by more specifically targeting CSC.

Finally, combining radiosensitizers with hypofractionated courses of radiation represents an attractive approach for increasing IR-induced killing of CSCs. For example, Brown et al. calculated the expected effect of the addition of etanidazole, a hypoxic radiosensitizer, with SABR and found equivalent NSCLC tumor cell killing with a modest single fraction SABR with etanidazole versus large multifraction doses of SABR without etanidazole (Brown et al. 2010). Such an approach has not been tested in the clinic.

4.5 Targeting Enhanced Telomerase Function

Essential for chromosomal integrity following multiple rounds of cell division, telomerase is present in 80–90 % of cancer cells but absent in the majority of somatic cells (Kim et al. 1994; Shay and Bacchetti 1997). Further experiments have shown that CSCs also express telomerase (Armanios and Greider 2005). Encouragingly, in vitro and in vivo disruption of telomerase (hTERT) has demonstrated tumor cell, and more specifically, CSC death (Hahn et al. 1999; Phatak et al. 2007). Also promising, when an oligonucleotide antagonist for the hTERT RNA template, Imetelstat, is used in vitro with esophageal cancer cells in combination with radiation, a synergistic and quantitative effect of double-stranded DNA breaks was observed (Wu et al. 2012).

Phase I clinical trials have begun, targeting the disruption of telomerase activity using Imetelstat. Currently, preliminary data has been reported by Thompson et al. for the use of Imetelstat in pediatric refractory or recurrent solid tumors. Thompson et al. administered Imetelstat as monotherapy to 20 pediatric patients with low toxicity, primarily myelosuppression, measurable for 17 children. Telomerase inhibition was observed in peripheral blood mononuclear cells in five of six children. Two children achieved a partial response to therapy (Thompson et al. 2013). While promising, further phase I and II evidence for Imetelstat, demonstrating safety and efficacy, is required. If proven safe, use of Imetelstat in combination with other therapies such as radiation, may be warranted.

4.6 Pro-survival Signaling as a Therapeutic Target

In the setting of cytotoxic treatment, CSCs utilize pro-survival signaling to overcome apoptosis. Pro-survival proteins expressed by some tumor cells are the class of inhibitor of apoptosis proteins (IAP), which bind and inhibit caspases, which are proteases essential for a cell to undergo apoptosis (LaCasse et al. 2008). In vitro and in vivo, inhibitors for X-linked IAP, a specific IAP associated with direct inhibition

of caspases and extrinsically signaled apoptosis, have been observed to increase radiosensitivity in CSC-like cells in NSCLC (Cao et al. 2004). While IAP inhibitors are being evaluated in clinical trials, to our knowledge the combination of IAP inhibitors and radiotherapy has not been tested.

4.7 Cancer Immunotherapy

Survival of CSCs also depends on evasion of the host immune system. CD47 is expressed by host cells and many CSCs inhibiting phagocytosis by interaction with the phagocyte signal regulatory protein-α (SIRP) (Willingham et al. 2012; Jaiswal et al. 2009). In vivo investigation with anti-CD47 antibody has demonstrated elimination of engraftment and complete remission of human acute lymphoblastic leukemia (ALL) in a mouse xenograft model (Chao et al. 2011). Combination immunotherapy with anti-CD47 and Rituximab, a monoclonal antibody to CD20, also demonstrated synergistic phagocytosis and cell killing of various non-Hodgkin lymphoma types (Chao et al. 2010). Demonstrating further clinical relevance, retrospective analysis demonstrated worse progression-free survival and overall survival in 200 ALL patients with increased CD47 expression (Soto-Pantoja et al. 2014).

Another class of immune modulators are the immune-checkpoint inhibitors, which are comprised of anti-CTLA4 antibody and PD-1/PD-L1 inhibitors (Sheridan 2014). With the advent of anti-CTLA4 antibody and PD-1/PD-L1 inhibitors, a synergy has been observed when combined with radiation in vivo, termed the "abscopal effect" (Verbrugge et al. 2012; Zeng et al. 2013; Postow et al. 2012; Golden et al. 2013; Hiniker et al. 2012a, b). Local radiation treatment combined with ipilimumab, an anti-CTLA4 antibody, resulting in response at untreated, metastatic tumor locations for melanoma and NSCLC via the abscopal effect (Golden et al. 2013; Postow et al. 2012; Hiniker et al. 2012a, b). Phase I/II results exploring ipilimumab with or without radiation for 70 patients with metastatic castration-resistant prostate cancer demonstrated modest results with complete response in one patient, partial response in two patients, and stable disease in six patients (Slovin et al. 2013). Finally, intratumoral injection of TLR9 agonists in combination with localized radiation therapy have demonstrated systemic responses to treatment in phase I/II trials for systemic low-grade B-cell lymphoma and cutaneous T-cell lymphoma, with 1 of 15 B-cell lymphoma patients achieving a complete response to treatment (Brody et al. 2010; Kim et al. 2012). While not fully robust yet, the ability for immunotherapy combined with local radiation therapy to cause complete and durable responses to treatment for metastatic cancer indicates that these approaches may be able to effectively ablate CSCs.

4.8 Modulating MicroRNA Pathways

MicroRNAs, which are relatively recent additions to our understanding of cancer biology, are now key players in the development of cancer (Babashah 2014). Moreover, modifying the levels of certain miRNAs such as miR-125, miR-155 and miR-146 appear to shift hematopoietic stem cells towards cancer stem-like cells leading to hematopoietic malignancy (O'Connell et al. 2008, 2010b; Zhao et al. 2011; Chaudhuri et al. 2012; So et al. 2014). Also, IR modulates levels of several miRNAs in vitro, an effect partially inhibited by the ROS scavenger cysteine (Simone et al. 2009). Modulation of miRNA function via synthetic miRNA derivatives, anti-miRNAs and pseudotarget sponges have been shown to modulate cancer pathways in vitro and in vivo using mouse models (Chaudhuri et al. 2011, 2012; Ebert et al. 2007; Lu et al. 2009). Human trials have been slow to develop due to challenges with bioavailability and concerns for toxicity, but phase I trials are now underway for MIRX34, a miR-34 derivative that inhibits p53, in advanced cancers with liver involvement. Combining future miRNA-targeted therapies with IR may be another approach for radiosensitizing CSCs.

4.9 Terminal Differentiation of CSCs

Acute promyelocytic leukemia is an aggressive leukemia where the hematopoietic system fills with rapidly dividing immature promyelocytes. It is; however, one of the most easily treatable types of cancer with a very good prognosis and low relapse rate. Treatment involves terminal differentiation of the cancer cells via administration of all-trans retinoic acid. Indeed, one aspect of CSCs that might make them especially refractory to treatment is their stemness- early developmental state with high self-renewal potential (Morrison et al. 2011). Cancers comprised of mature cell types such as mature teratomas are thus usually easily resectable without need for adjuvant therapy and with low rates of relapse. Thus, strategies to induce differentiation of CSCs may lead to more durable treatment responses.

5 Conclusion

In many tumor types, CSCs have intrinsically greater resistance to ionizing radiation than the remaining cancer cells, as demonstrated by both in vitro and in vivo experiments. This represents a major obstacle, because if not completely ablated by therapy, CSCs are capable of regenerating tumors and leading to cancer recurrence. CSC radioresistance thus represents an important clinical problem. Here we have discussed the mechanisms that CSCs employ to resist IR and therapeutic strategies that are currently being used in the clinic, are under development, or are on the horizon for overcoming these.

CSC mechanisms for IR evasion include up-regulation of ROS scavengers such as glutathione, improved DNA repair mechanisms, robust checkpoint signaling, enhanced telomerase function, and increased expression of pro-survival signaling pathways. Local tumor hypoxia can further contribute to CSC radioresistance. Potential methods for combating these include improved microscopic cancer surveillance, hypofractionated radiotherapy, use of radiosensitizing agents, checkpoint signal inhibition, modulation of cell survival pathways, cancer immunotherapy, and induction of CSC differentiation. Some of these methods, such as hypofractionated radiotherapy and chemotherapy-based radiosensitizers are currently in clinical practice and have been shown to be effective in improving tumor control and patient outcomes in the appropriate clinical settings. Others, such as cancer immunotherapy, have shown early clinical and pre-clinical success in combination with therapeutic IR, although further studies are required to fully evaluate effectiveness and appropriate clinical uses. Still others, such as modulation of pro-survival and checkpoint signaling proteins, microRNA therapy, and inhibition of telomerase function have shown some promising results in vitro and in mouse models, but translation to the clinical setting has been technically challenging (Babashah and Soleimani 2011; Babashah 2014).

Indeed, the effective targeting of CSCs in cancer therapeutics will require a comprehensive understanding of the resistance mechanisms employed. While preclinical and clinical investigations have identified numerous potential strategies for combating CSC resistance to therapy, further studies are still required. The future looks bright; however, with several preclinical studies and clinical trials in progress. Ultimately, completion of positive clinical trials will be required to bring these approaches into routine clinical practice.

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Chapter 19
Therapeutic Implications of Cancer Stem Cell: Challenges and Opportunities in Translational Studies

Chunguang Yang, Kunlin Jin, Yue Zhang, Hong Sun, and William C. Cho

Abstract Cancer stem cells (CSCs) are rare in cancer population, but they can reconstruct the whole bulk of tumor if most of the cancer cells are killed by radiotherapy or chemotherapy. However, the mechanism of CSCs' self-renewal, regeneration and therapeutic resistance is still unknown. Here we discuss the role of CSCs in cancer relapse and progression, as well as their diagnostic and therapeutic potentials. We also confer the biomarkers in pre-clinical models and clinical trials to evaluate the therapeutic effectiveness of CSCs. In addition, we discuss the potential therapeutic strategies of eradicating CSCs in tumors, such as cytotoxic, radiation, signaling pathway and differentiation therapies.

Keywords Cancer stem cells • Biomarkers • Diagnosis • Clinical outcome • Clinical trials • Therapy

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1 Introduction

Cancer is considered as a cellular disorder, which has strong capacity of proliferation and hard to be completely eradiated by chemotherapy, radiotherapy and surgery because of its properties of invasion and metastasis. Since 1997, cancer stem cells (CSCs), a rare number of population in cancer cells, have properties of self-renewal, potential strong proliferation and resistant to radio/chemotherapy (Reya et al. 2001).

Like stem cells in normal organs and tissues, CSCs have similar cell behaviors to normal stem cells (Avital et al. 2014). The links between stem cells and CSCs are vague. Some studies suggested that normal stem cells could transform to cells possessing of cancer-like characteristics or cancer cells in vitro and in vivo, such as methylation alterations and tumorigenicity of cancer cells (Hentze et al. 2009; Gropp et al. 2012; Zhu et al. 2015). CSCs can self-renew and differentiate into cancer progenitor cells through asymmetric replication (Adikrisna et al. 2012; Ginestier et al. 2007). On the contrary, CSCs can generate from the dedifferentiated or acquired stem-like progenitor cells via signaling or microenvironment alteration (Jamieson et al. 2004; Kum et al. 2012). In addition, other evidences showed that CSCs can derive from cancer cell transformation, through epithelia-mesenchymal transition (EMT), gene mutation, and enzymatic activation (Ginestier et al. 2007; Huang et al. 2009; Liang et al. 2010; Proia et al. 2011).

Although there is no single marker to identify CSCs, there are several markers for identification of the CSCs, including CD133, CD34, CD44, CD24, CD117, epithelial-specific antigen (ESA) and aldehyde dehydrogenase (ALDH) (Table 19.1).

Table 19.1 Biomarkers of cancer stem cells in many cancers

Tumor type	Cell surface marker(s)	Reference(s)
Acute myeloid leukemia	$CD34^+CD38^-$	Bonnet and Dick 1997
Brain tumor	$CD133^+$	Singh et al. 2003; 2004
Breast cancer	$CD44^+CD24^{-/low}Lineage^-$	Al-Hajj et al. 2003
Colon cancer	$CD133^+$	O'Brien et al. 2007; Ricci-Vitiani et al. 2007
Colorectal cancer	$ALDH^+$, $CD44^+$	Huang et al. 2009
Ewing's sarcoma	$CD133^+$	Suva et al. 2009
Head and neck cancer	$CD44^+Lineage^-$	Prince et al. 2007
Liver cancer	$CD90^+CD45^-$ ($CD44^+$)	Yang et al. 2008
Lung cancer	$SP\text{-}C^+CCA^+$	Kim et al. 2005
	$CD24^+$, $CD44^+$, $CD133^+$	Eramo et al. 2008
	SP	Ho et al. 2007
Melanoma	$ABCB5^+$	Schatton et al. 2008
Ovarian cancer	$CD44^+CD117^+$	Zhang et al. 2008b
Pancreatic cancer	$CD44^+CD24^+ESA^+$,	Li et al. 2007
	$CD133^+$	Hermann et al. 2007
Prostate cancer	$CD44^+\alpha_2\beta_1{}^{hi}CD133^+$	Collins et al. 2005
Retinoblastoma	$ABCG2^+$	Seigel et al. 2005

Though traditional therapies could deplete some of the cancer cells, there is not a very effective method to eliminate CSCs. Some drugs or antibodies such as rituximab and cetuximab have been used in pre-clinical or clinical trial for targeting CSCs or lineage of lymph cells, but the clinical prognosis is still not steady (Schlaak et al. 2012; Fan et al. 2013). Therefore, it is very desirable to develop novel, more effective treatments targeting CSCs to treat cancer.

2 Role of Cancer Stem Cells in Cancer Relapse and Progression

Cancers often recur in a frame of time after treatment; especially the advanced cancers relapse in shorter time. Ginestier et al. (2007) have demonstrated that aldehyde dehydrogenase 1 (ALDH1) expression in patients with breast cancer is an independent factor affecting clinical prognostic factors, such as Ki-67 status, tumor size, and histological grade. Patients with tumors having ALDH1-positive staining had a poorer prognosis, compared to patients with tumors with ALDH1-negative staining. A large proportion of patients with cancer deaths are caused by recurrent tumors, but the cellular and molecular mechanisms underlying tumor relapse remain unknown. The expression of EphB2 (Wnt target gene, the receptor tyrosine kinase EphB2) gene in intestinal stem cells (ISCs) is strongly associated with metastatic colon cancer based on Gene Set Enrichment Analysis (GSEA). Thirty-eight percentage of the EphB2^{hi}ISC genes are overexpressed in colorectal cancer (CRC), including the ISC markers Lgr5 and Ascl2, yet, other intestinal differentiation markers are down-regulated. Patients bearing CRCs with high expression of ISC genes had a risk of relapse tenfold higher than those with low levels, and EphB2-ISC signature is a predictor of disease relapse independent of AJCC (American Joint Cancer Committee) staging using the Cox Proportional Hazards Model demonstrated (Merlos-Suarez et al. 2011).

The phenomenon was also identified in mouse orthotropic implantation model, in which CXCR4^{+}CD133^{+} CSCs were mainly determined for tumor metastasis (Hermann et al. 2007). Although more efforts have been made to improve diagnosis, treatment and prognosis of cancers, there were scarce or no biomarkers for the early diagnosis. Therefore, specifically targeting CSCs may be more useful to eradicate the tumors through combination therapies for overcoming chemoresistance and inhibiting relapse or metastasis. MUC4 knockdown in combination with gemcitabine to inhibit side population (SP) or CD133 may eradicate cancer mass and thereby prevent prostate relapse (Mimeault et al. 2010). Targeting certain biomarkers, such as CD133, are able to reduce cancer progression (Skubitz et al. 2013). However, there are a few of stem cell markers and it is unclear whether these markers associate with the recurrence and radio/chemotherapy, as some CSC markers were up-regulated after bearing radio/chemotherapy and others were down-regulated. For example, in ovarian cancer, CD133, ALDH1A1 (aldehyde dehydrogenase 1 A1), CD44 were

investigated in tumors from recurrent platinum-resistant patient, and only CD133 was significantly increased compared with matched primary ovarian tumors (Steg et al. 2012).

3 Diagnostic Potential of Cancer Stem Cells in Oncology

Most cancer cases are diagnosed based on disease history, clinical etiology, radiation scan, MRI, pathomorphology, cytological examination, immunophenotyping, cytogenetic analysis of blood and bone marrow. In past decades, a few biomarkers, such as AFP, APC, CEA, have been used to determine the cancer grades and the prognosis. In nowadays, some new technologies are used to diagnose the cancers or CSCs, including gene diagnosis, high-throughput screening of gene-chips, protein-chips (Gupta et al. 2009; Hartwell et al. 2013). But whether CSCs play an important role in cancer diagnosis remains unknown.

In glioma, expression levels of nestin and CD133 were significantly higher as the glioma grade advanced, and the survival rate of patients with nestin$^+$/CD133$^+$ expression was lower than the patients with negative expression of these proteins. In the contrast, the patients with low expression of these two markers were associated with long survival rate. Thereby, combination detection of nestin/CD133 co-expression might be diagnostic significance to predict the aggressive nature of glioma (Zhang et al. 2008a).

Expression of ALDH1 was proved an independent factor for predicting early metastasis and lower survival rate of the patients with inflammatory breast cancer (IBC). The data suggested that CSC component, such as ALDH enzymatic activity, might mediate the metastatic and aggressive behaviors of IBC. ALDH1 expression represented a possible marker of CSCs at diagnosis to predict patient outcome in IBC (Charafe-Jauffret et al. 2010).

CD34$^+$CD38$^-$ cells have been used to identify as CSCs in leukemia, and the number of these cells affected the prognosis of leukemia. For example, van Rhenen et al. (2005) demonstrated that patients with high rate of CD34$^+$CD38$^-$ CSCs were significantly correlated with a high minimal residual disease (MRD) frequency and poor survival. Therefore, it was considered that a large number of CD34$^+$CD38$^-$ cells at diagnosis had a higher percentage of chemotherapy resistant cells, which, in turn, resulted into the outgrowth of MRD and poorer clinical outcome.

Although several CSC markers play critical roles in predicting the prognosis and diagnose in some advanced cancers, it remain lacking specific biomarkers of CSCs for early diagnosis of cancers. Considering both the genetic alterations and differentiation states of CSCs, it is possible that individualized diagnosis and treatment in different patients are performed.

4 Cancer Stem Cells as Biomarkers to Predict Clinical Outcome

Clinical outcomes of cancers are known to be classified by clinical disease, pathology differentiated types, lymph nodes and distant metastasis. Many studies suggested that CSCs are associated with cancer clinical outcome. However, different primary cancers may have different CSC biomarkers. Therefore, whether CSC biomarkers can be used to predict clinical outcome remains largely unknown. In ovarian cancer, some of these CSC markers including CD44, CD133, Hoechst-excluding cells (the SP) and ALDH1A1, have been identified are associated with poor clinical outcomes (Szotek et al. 2006; Slomiany et al. 2009; Landen et al. 2010; Silva et al. 2011). In leukemia, $CD34^{+}CD38^{-}$ expresses in leukemia stem cells (LSCs) and predicts the patient survival. It has been proved that LSCs had 'stemness' properties and was highly significant independent predictor of patient survival, which influenced the clinical outcome of acute myeloid leukemia (AML) (Eppert et al. 2011). In addition, these markers had similar properties to other cancers such as breast, colon, brain and pancreas cancers (Ginestier et al. 2007). In head and neck squamous cell carcinoma (HNSCC), it was determined that some CD44 isoforms mediated proliferation, migration and cisplatin sensitivity and were associated with advanced T stage (variant 3 and v6), distant metastasis, radiation failure (v10), and perineural invasion (v6), compared with primary tumors (Wang et al. 2009). In contract, CD44v3 were associated with distant metastasis in colon carcinoma (Kuniyasu et al. 2001) and CD44 variants mediated tumor cell migration (Bourguignon et al. 1998). Some CSCs are derived from stratified tissues. For example, ISCs belonged part of the luminal cells and most of stem-like cell population positioned at the bottom of tumor structures, which were reminiscent of crypts in both normal and cancer tissues. These cells were marked by EphB2 and Lgr5, purified by FACS (fluorescence activated cell sorting) and implanted into immunodeficient mice to identify possessing self-renewal potential. These ISC-like cells were association with clinical outcome (Merlos-Suarez et al. 2011).

CSCs can influence the biological behaviors of cancers. Some colon CSC genes were differently relevant to risk of relapse, including CD44, ALCAM, DTX2, HSPA9, CCNA2, PDX1, MYST1, COL1A1 and ABCG2 (Giampieri et al. 2013). Bmi1 protein contributes to self-renewal of $CD133^{+}$ populations and regulates proliferation and determines cell fate of $CD133^{-}$ populations of primary human glioblastoma (GBM) cell lines. Furthermore, Bmi1 expression was predictive of clinical outcome of GBM patients (Venugopal et al. 2012).

Meta-analysis has evaluated the expression of CD133 and epithelial cell adhesion molecule and has determined whether CSCs are as prognostic factors and association with clinical and pathologic features of hepatocellular carcinoma (HCC).

The analyses show that the presence of CSCs is significantly associated with a poor histological grade, the elevated serum alpha-fetoprotein level, poor survival, including overall survival and disease-free survival. But, there are no significant relations between the presence of CSCs and tumor size, tumor stage, hepatitis and cirrhosis. It is also found that CD133 plays a significant role in predicting the clinical outcome (Ma et al. 2013). Studies have demonstrated that DNA methylation is associated with prognosis of patients with cancers. To determine the correlation between normal stem cells and CSCs, Zhuang et al. (2012) investigated the most comprehensive study of DNA methylation changes through analyzing 27,578 CpGs in 1475 samples, including normal cells in advance of non-invasive neoplastic transformation tissues and tissues of non-invasive cancers, invasive cancers and metastatic cancers. They found that stem cell polypomb group target genes (PCGTs) in normal cells in advance of the first morphological neoplastic changes and hypomethylation of embryonic stem cells are increased significantly with invasion in both the epithelial and stromal tumor compartments of cancers. Yet, embryonic stem cells (ESCs) hypomethylation progressed significantly from primary cancer to metastatic cancer and defined a poor prognostic signature in four different gynecological cancers.

5 Therapeutic Implications of Cancer Stem Cells

The population died from cancer is significantly increased and is the first cause of fatality rate in developing countries and the second reason for death in developed countries. Because CSCs are resistant to conventional therapies, it is very urgently needed to find a novel strategy to eradicate cancers from patients through targeting CSCs.

However, few CSCs are sensitive to specific inhibitors of signaling pathways, so targeting these CSCs to kill the whole masses of cancer may be not feasible. Some CSCs are resistant to or insensitive to inhibitors of cell signaling, so combination signaling blockers and drugs or radiotherapy may be effective to dispel cancers or prevent cancers from relapse. Cell cycle checkpoint kinase 1 (Chk1) is an integral component of the DNA damage response mediating cell cycle arrest and DNA damage repair. Venkatesha and colleagues (2012) tested CD24−, CD44−, and ESA-positive CSCs by using two patient-derived pancreatic cancer cell lines for drug sensitive tests in vitro and in xenograft models. They investigated the percentage of CSCs in cancer cells and CSC tumor-initiating capacity after treatment with the Chk1 inhibitor, AZD7762 and gemcitabine. They found that CSCs were significantly reduced by the combination of AZD7762 and gemcitabine. Interestingly, secondary tumor initiation was significantly delayed in response to primary tumor treatment with AZD7762 and gemcitabine compared with control, AZD7762 or gemcitabine alone. Zhang and colleagues (2010) found that CSCs enriched in mammospheres as compared to primary tumor cells, in parallel with increased Akt signaling, and activation of the canonical Wnt/β-catenin pathway. When inhibiting the Akt pathway, the canonical Wnt signaling was as well as inhibited, in addition,

sensitizing CSCs to ionizing radiation treatment. Thus, these results suggested that combination signaling and ionizing radiation treatment may be of potential therapeutic benefit to patients. Other studies show that targeting death receptor 5 (DR5), sonic hedgehog (SHH) or mammalian target of rapamycin (mTOR) in combination with gemcitabine result in pancreatic CSCs sensitive to gemcitabine and CSCs decrease (Mueller et al. 2009). So it is implicated to combine signaling pathways and other methods to target the CSCs in order to treat cancer.

It is found that some CSCs are sensitive to DNA-damaging, and therefore, special drugs, DNA-damaging agents and DNA-damaging radiation may be useful for cancer therapy. Venkatesha and colleagues (2012) found that pS345 Chk1, a DNA damage marker, was induced in CSCs, but not in non-CSC cells. These data suggest that Chk1-mediated DNA damage response is greater in CSCs than in non–CSC cells, and demonstrate that Chk1 inhibition may selectively sensitize pancreatic CSCs to DNA-damage agent, which may be a potential therapeutic strategy for cancer therapy.

But, it is found that CSCs ($CD44^+/CD24^-$ and $CD133^+$ cells) are significantly more resistant to DNA damage drugs than non-CSCs or CSCs depleted populations, and CSCs are sensitized to the drugs by the heat shock protein-90 inhibitor 17-dimethylaminoethylamino-17-demethoxygeldanamycinhydrochloride (17-DMAG) in breast cancer. These CSCs are co-expressed other stem cell genes, including Oct4, Notch1, Aldh1, and Sox1, suggesting that targeting these biomarkers can eradicate CSCs (Wright et al. 2008).

Dallas and colleagues used 5-fluorouracil (5-FU) and oxaliplatin to develop chemoresistant cell line, and used insulin-like growth factor-I receptor (IGF-IR) monoclonal antibody (mAb) AVE-1642 to inhibit signaling of these induced cells in vitro and in vivo. The resistant cells were enriched $CD133^+/CD44^+$ cells by 5- to 22-fold, along with increase in phosphorylated and total IGF-IR levels. These cells were fivefold more responsive to IGF-IR inhibition relative to parental cells in vitro. The results demonstrate that chemotherapy-induced IGF-IR activation provide for enhanced sensitivity to IGF-IR–targeted therapy, which may be a novel therapeutic approach to treat cancer (Dallas et al. 2009).

6 Development and Use of Appropriate Pre-clinical Models for Evaluation of Drug Efficacy Against Cancer Stem Cells

Several studies have documented that CSCs are resistant to some conventional clinical drugs, such as paclitaxel, cisplatin and gemcitabine (Rutella et al. 2009; Venkatesha et al. 2012). Up to now, the gold standard to evaluate the tumorigenicity of CSCs is implanting them into immunodeficient mice. Firstly, the number of CSCs has 100 times less than non-CSCs to form tumors. Secondly, they can potentially differentiate to the primary tumors and have capability of self-renewal, without loss of tumorigenic potential in order to be re-transplanted serially through subcutaneous, intraperitoneal or orthotropic injection (Bonnet and Dick 1997; Singh et al. 2004;

Ricci-Vitiani et al. 2007; Zhang et al. 2008b). Therefore, evaluating drug efficacy against CSCs should be not only tested in vitro, but also in animal bodies. In addition, the drugs used in animals should have good efficacy against cancer cells and have a few or none side effects to be applied appropriately in human. For example, the mTOR inhibitor, rapamycin, deforolimus or everolimus used in mice has negligible systemic toxicity in pre-clinical experiments (Bhola et al. 2010). In early clinical trials, everolimus has great efficacy of anti-tumors including in non-small cell lung cancer, breast cancer, renal-cell carcinoma and hematologic malignancies, and has the low toxicity, such as mucositis, asthenia, hyperlipidemia and neutropenia through evaluation of patients by assessment of history, physical examination, complete blood count, hepatic and renal function tests and so on (Hudes et al. 2007; Bissler et al. 2008; Rizzieri et al. 2008; Ramalingam et al. 2010).

However, it is required that drugs should selectively target CSCs to kill the whole cancers. Combination signaling inhibitors and chemotherapy or radiotherapy can enhance their synergetic efficacy and may be an effective approach to kill cancer cells and CSCs. For instance, in breast cancer, combination rapamycin and paclitaxel, carboplatin, docetaxel, and vinorelbine can enhance cancer cells sensitive to chemotherapeutic cytotoxicity and apoptosis (Mondesire et al. 2004; Zhang et al. 2012). In leukemia, combination of Wnt/β-catenin, hedgehog pathway components, transforming growth factor-β (TGF-β) and Janus kinase 2 can sensitize BCR-ABL inhibitors against leukemic stem cells (Al Baghdadi et al. 2012). Zhou et al. (2013) demonstrated that combining N-(2-hydroxypropyl) methacrylamide (HPMA) copolymer-cyclopamine conjugate (P–CYP) with HPMA copolymer-docetaxel conjugate (P–DTX) selectively killed cancer stem/progenitor cells. Furthermore, combination of drugs and radiation also removes CSCs. For example, in glioblastoma, TGF-βR-I kinase inhibitor, LY2109761, enhances CSCs radiation sensitivity (Zhang et al. 2011). Chloroquine (CQ) promotes γ-irradiation (γ-IR) against cancer cells and has strongly therapeutic benefits to kill highly stem-like glioma cells (SLGCs), which has radioresistance induced by γ-IR (Firat et al. 2012).

Combination of two or more signaling pathways to inhibit CSCs growth to evaluate the efficacy of signaling inhibition drugs may be also a good pre-clinical model for against CSCs. For instance, the IGF-1R inhibitor, picropodophyllin, not only suppresses CSC features, including mammosphere formation in vitro and tumorigenicity in vivo, but also reduces phospho-Akt, $ALDH^+$ breast CSCs and inhibits the $CD24^-CD44^+$ breast CSC EMT. In addition, rapamycin inhibits breast CSCs in vitro and in vivo (Chang et al. 2013). These give us an idea that signaling pathways have crosstalk with each other, and activation of these signaling pathways may show the proliferation of CSCs. So some drugs that inhibit specific signaling pathway may also down-regulate other signaling pathways, which can be used to evaluate pre-clinical tests especially finding specific drugs against CSCs.

7 Cytotoxic Therapy

CSCs may hide somewhere deeply in cancer cells, which are needed to discover by biomarkers. The biomarkers can be used to mark CSCs in the cancer, which supports the specific cytotoxic therapy through targeting CSCs to treat cancers. However, the strategy must target specifically CSCs, but not normal stem cells. Otherwise, it leads both normal tissue cells and cancer cells dead.

Despite the CSCs are only in a minority of the cancer cells, targeting the CSCs can efficiently prohibit cancer growth and tumor formation. Several experiments document that anti-CSCs could inhibit cancer cell growth and tumor initiation in vitro and in vivo. Both in head and neck cancer and breast cancer, the anti-CD133 targeted toxin, dCD133KDEL, can significantly inhibit sorted $CD133^+$ cancer cell growth and colony formation, but not $CD133^-$ cells. When dCD133KDEL is used to treat sorting $CD133^+$ cells implanted in mice, it show remarkable anticancer effects of inhibiting the growth of the tumors (Waldron et al. 2011; Skubitz et al. 2013). However, the dCD133KDEL may not completely kill the $CD133^+$ cancer cells and stop the tumor further growth, suggesting that the CD133– positive cells are only portion of the CSCs.

Other therapeutic implications for treatment of cancers include the combination of pathways and cytotoxic therapy to enhance treatment efficacy. For example, targeting epidermal growth factor receptor (EGFR), pAkt, NF-κB or MIC-1 induce disintegration of SP cell-derived spheres and reduce the viability of SP and non-SP cell fractions and enhance their sensibility to the cytotoxic effects of docetaxel in prostate cancer cells (Mimeault et al. 2012).

As concerned above, cytotoxic therapy targeting CSCs may be an efficient avenue to treat cancer, without impairing normal stem cells and differentiated tissue cells. Although some studies have been performed, such as in head and neck cancer (Waldron et al. 2011), it needs more selective drugs to be cytotoxic to CSCs.

8 Radiotherapy

Many therapies are applied in clinical treatment for patients with cancers following advanced technologies, such as surgery, chemotherapy, radiotherapies, radiofrequency therapy, and combination two or three of them. However, the overall therapeutic benefits are still inefficient especially, when patients with advanced cancers and the mortality of the people with cancers remains high. The radiotherapy targeting the CSCs may be an efficient and specific strategy.

Although radiotherapy has been used in clinical treatments for patients with cancers, and some kinds of cancers are sensitive to radiation therapy, which leads a higher survival in the early stages of the cancer progress, such as in nasopharyngeal carcinoma (Lo et al. 2004), CSCs are more resistant to radio/chemotherapy than non-CSCs (Diehn and Clarke 2006; Murat et al. 2008), which in turn causes the

severe problem of cancer treatment. Even more, radiotherapy may induce cancer cells to possess some properties of stem-like cells (Ghisolfi et al. 2012).

Some receptors express in surface of CSCs, which augment the signaling responsive to ligands causing the activation of downstream signaling pathways, abnormal proliferation and resistance to radiotherapy. It is proved that the expression of CD133 and EGFR is associated with poor survival of patients with cancer chemo/radiotherapy. Therefore, not only targeting CSCs but also inhibiting EGFR and blocking CSC signaling to crosstalk each other and to be amplified, can reverse the cancer resistant to conventional therapy and improve a higher survival rate (Murat et al. 2008).

Signaling pathways are abnormally activated in CSCs and participate in modulating CSCs resistant to radiotherapy. For example, in medulloblastoma, cancer cells transform to stem-like potency cells through activation of Akt signaling and PTEN loss. Inhibition of Akt signaling makes CSCs in the perivascular region sensitive to radiation, and thus causes CSCs easier to apoptosis, compared to non-CSCs (Hambardzumyan et al. 2008). In GBM, TGF-β modulates CSC radiotherapy. When TGF-β receptor (TGF-βR) I kinase inhibitor, LY2109761, in combination with radiation are used in CSC therapy, it increases GBM stem cell radiosensitivity and augments the tumor growth delay in vivo (Zhang et al. 2011).

Another study investigated in malignant glioma that radiation enhances virotherapy toxic to CSCs. When malignant glioma stem cells are treated with oncolytic adenovirus, CRAd-Survivin-pk7, and radiation, the CSC growth are significantly inhibited in vitro and in vivo (Nandi et al. 2008).

Accordingly, combinations of signaling pathway inhibitors, cell surface receptors blockade, DNA damage, cell cycle checkpoint kinases and radiotherapy targeting CSCs may eradicate cancers.

9 Targeting Stem Cell Pathways

There are many pathways associated with CSCs, some important ones are discussed as below.

Notch signaling presents in cells from invertebrate to vertebrate organisms, and is an evolutionarily highly conserved cell signaling. Different organism cells communicating to each other use the signaling pathway to achieve unique developmental goals (Artavanis-Tsakonas et al. 1999). Notch signaling contains four transmembrane Notch receptors (Notch 1–4) and five ligands (Delta-like [Dll]-1, -3, and -4, and Jagged-1 and -2). When Notch ligands-receptors compounds are cleaved by proteolytic γ-secretase, Notch intracellular domain of Notch (NICD) will be released and then is translocated in the nucleus and interacts with the DNA-binding protein G protein CSL (CBF, suppressor of hairless, LAG-1; also referred to as RBP-Jκ) that interacts with corepressor complexes transcriptional repressors and targets the Hairy/Enhancer of split (Hes-1 to -7) and Hey (Hey-1, -2, -L) (Stockhausen et al. 2010).

Wnt/β-catenin signaling acts through the Disheveled protein, LRP5/6 co-receptors and Frizzled receptors, and then trigger downstream events, leading to the inactivation of this β-catenin-degradation complex, containing Axin, APC (adenomatous polyposis coli) and glycogen synthase kinase 3 (GSK3). GSK3 blockade activates β-catenin stabilization and transcription, which is mediated by TCF proteins or affects HIPK2. During the activation events, β-catenin is the chief downstream activation, and enters the nucleus to activate leading downstream transcription. In the normal steady state, β-catenin is degraded by phosphorylation at serine and threonine residues through the activation of casein kinase Iα and GSK3 (MacDonald et al. 2009; Sokol 2011).

PI3K/Akt/mTOR cascade pathway is comprised of ligands of PI3K, receptor of PI3K, activating PI3K, serine/ threonine protein kinase B (Akt/PKB), mTOR, transcription factors. The receptors of PI3K include vascular endothelial growth factor, insulin like growth factor receptor and platelet-derived growth factor (Franke et al. 1995; Crowder and Freeman 1998), of which activation regulates Akt/PKB, following mTOR phosphorylated, and then regulates downstream transcription factors such as p70 S6 kinase and 4E-BP1 phosphorylation, which cases cell growth and proliferation (Nave et al. 1999; Burroughs et al. 2003). During the process of activation of PI3K/Akt/mTOR, Akt/PKB is inactivated by the tumor suppressor gene PTEN (Marty et al. 2008; Squarize et al. 2013).

10 Differentiation Therapy

Growing evidence has proved that cancer cells occur to EMT, which renders them to transform to CSCs capable of invasion, metastasis, and recurrence (Kalluri and Weinberg 2009). It is observed that inducing differentiated normal mammary epithelial cells to undergo EMT will generate epithelial stem-like cells. Even more, EMT can promote differentiated neoplastic cells to generate CSC. EMT is associated with mouse mammary stem cells, normal human breast stem-like cells, and neoplastic human breast stem- like cells (Mani et al. 2008). However, whether the CSCs are transited from the cancer cells or adult tissue cells is still poorly understood.

When cancer cells diffuse in other organs to achieve distant metastasis, these cells must escape or break the barrier of stable in situ, such as the epithelial cell polarity, including polarized cytoskeletal and plasma membrane proteins, E-cadherin, CD24 and other proteins.

The cancer cells are gradually transited to possess stem-like or mesenchymal capacity, which are CSCs. During these times, many different transcription factors, signaling pathways and chromatin regulators are activated (Humbert et al. 2008; Zhao et al. 2008). Hence, inhibition of epithelial cancer cells transiting to mesenchymal cancer cells or depletion of CSC pool may be therapeutic strategies to treat cancer.

11 Challenges in Designing Clinical Trials with Stem Cell-Directed Therapies

Relapse is a major factor of cancer death and CSCs play the critical role in recurrence, so marking the CSCs to kill them to avoid relapse may be a director for CSC therapies.

Perumal et al. (2012) investigated 360 lung adenocarcinoma patients and four non-small cell lung cancer (NSCLC) lines, they found that one gene, FUS, was significantly down-regulated in chromosomal translocation and four genes (TOP2A, AURKB, BRRN1, CDK1) were significantly up-regulated in chromosome condensation pathway in which genes were likely associated with stem-like properties and might predict survival. This identifies that a gene signature is related with poor prognosis and may be designed to stem cell-directed therapies for patients with cancers filled with many CSCs (Perumal et al. 2012). In AML, when compared with AML $CD34^-$ cells and normal $CD34^+$ bone marrow (BM) cells, three genes, ankyrin repeat domain 28 (ANKRD28), guanine nucleotide binding protein, alpha 15 (GNA15) and UDP-glucose pyrophosphorylase 2 (UGP2), in AML $CD34^+$ cells, have a high transcript level which are related with a significant poorer overall survival (OS) (de Jonge et al. 2011). So targeting these genes in CSCs may be a directed strategy for cancer therapy, but up to now there is no selective targeting gene to kill cancer cells.

12 Interpreting Clinical Trials to Evaluate Cancer Stem Cells Targeting Therapies

Many clinical trials have been performed using targeting CSC drugs to find a very powerful strategy to treat cancers. But the mechanisms underlying effecting CSC growth, division, proliferation and radiochemotherapy resistance are still elusive. Therefore, interpreting clinical trials and finding new novel methods to evaluate targeting CSC therapies is highly required.

Chronic myeloid leukemia (CML) is a cancer driven by the BCR–ABL1 oncogenic protein, which activates tyrosine kinase. Thus, tyrosine kinase inhibitors (TKIs), including imatinib mesylate (IM), nilotinib and dasatinib can be efficient to inhibit proliferation of CML stem cells but do not induce apoptosis (Druker et al. 2001). In addition, imatinib discontinuation leads patients with CML, two of four patients, to sustain complete molecular remission after 14 and 15 months from discontinuation (Merante et al. 2005). However, quiescent CML stem cells, even knocked out the kinase activity of BCR-ABL, are insensitive to TKI. It shows that just anti-proliferation therapy against CML not the leukemia CSCs cannot kill all

leukemia cells (Jorgensen and Holyoake 2007). So preferentially targeting quiescent leukemia stem cells might be able to treat leukemia, such as lonafarnib, which experimented in vitro is needed to study in clinical trials (Jorgensen et al. 2005). In small cell lung cancer (SCLC), CD133 positive cancer cells exhibit an increased expression of the mitogenic neuropeptide receptors. And a novel neuropeptide antagonist, peptide-1, could inhibit the CSC growth and promote its proapoptotic effects. Peptide-1 is an analogue of SP-G (substance P analogue) that has been completed a phase I clinical trial for SCLC (Sarvi et al. 2014).

As CSCs undergo EMT to sustain their capability of invasion and metastasis. In advanced HCC, Livraghi et al. (2005) applied a product containing stem cells differentiation stage factors (SCDSF) to inhibit tumor growth in vitro, in vivo and in patients. They assessed efficacy of SCDSF in patients with HCC who, total of 179, were not suitable for resection, transplantation, ablation therapy, or arterial chemoembolization. The result shows that a significant difference survival between the group of patients who respond to treatment versus the control group of patients. The study indicates the efficacy of differentiation therapy in patients with advanced HCC, in which many CSCs might exist.

Cancer cells especially CSCs have the capability of sustaining angiogenesis to get nutrition from blood vessels, in which vascular endothelial growth factor plays the key role. Then clinical trials of anti-vascular endothelial growth factor antibody are contrived to remiss the patients with metastatic renal cancer. The phase II study of bevacizumab for renal cancer selective treatment shows that patients in treatment group have longer progression-free survival than in control group, which might be explained that the potential of angiogenesis was inhibited in CSCs (Yang et al. 2003).

Clinical studies should not only control tumors and efficiently inhibit CSCs, but also need to investigate the specific mechanisms of CSCs.

13 Conclusions and Future Perspectives

CSCs consist of a sparse number of cancer cells, which play an important role in caner growth, proliferation, differentiation, self-renewal, radio/chemotherapy resistance, metastasis, recurrence and patient mortality (Alisi et al. 2013). Although increasing number of biomarkers are identified for the CSCs, there are still few reliable biomarkers to detect all of the CSCs in different cancers. On the other hand, there are many drugs, inhibitors and monoclonal antibody agents used in vitro, in vivo and in clinical trials to target CSCs (Adikrisna et al. 2012; Yamashita et al. 2013).

It is a trend that some combination methods to eradicate as many cancer cells as possible (Newell et al. 2009; Bandyopadhyay et al. 2010; Ramalingam et al. 2010). For example, combination of targeting EGFR, pAkt, NF-κB and MIC-1 are effective in suppressing the SP cell formation and inducing disintegration of SP cell-derived spheres and decreasing the viability of SP and non-SP cell fractions. Moreover, targeting of these oncogenic products can induce chemoresistant SP cells to apoptosis and make them more sensitive to cytotoxicity (Mimeault et al. 2012). And down-regulation the mucin MUC4 can enhance $CD133^+$ pancreatic cells sensitive to chemotherapeutic drug, gemcitabine and its cytotoxic effects (Mimeault et al. 2010). In glioma, Notch inhibitors do not alter the glioma stem cells responsive to radiation inductive DNA damage but just sensitize them to radiation (Wang et al. 2010). And inhibitors of Chk1 and Chk2 checkpoint kinases can reverse radioresistance of $CD133^+$ glioma stem cells (Bao et al. 2006). So combining Chk1 and Chk2 checkpoint kinases with Notch inhibitors might improve their efficacy against CSCs. In addition, vascularization angiogenesis is also a property of CSCs critical to tumor formation and maintenance. Targeting proteinYKL-40, a 40 kDa secreted glycoprotein discovered as a heparin-binding protein, and ionizing irradiation can synergistically inhibit tumor vascularization and malignancy (Shao et al. 2014). Another concept of tumor therapy is hyperthermia therapy performed efficiently (Nikfarjam et al. 2005), and normal stem cells are more sensitive to it than CSCs through this therapy (Murphy and Richman 1989; Wierenga et al. 2003), but it in combination with radiotherapy and some chemotherapies show more powerful to kill cancers (Wierenga et al. 1998; Wust et al. 2002). However, these therapies are not yet lethal for CSCs, and widespread clinical use of these therapies have been limited by their diffuse heating which are toxic to non-tumor tissues and limited by relative thermal ablative instrumentations. Hence then, development of some new minimally invasive and highly localized nanotube-mediated thermal materials to treat CSCs has been applied (Atkinson et al. 2010; Burke et al. 2012).

CSCs may come from parts of the cancer cells and undergo some processes, such as from root to stem, to branches, to leaves and to falling leaves (Sell 2004). Thus, here we hypothersize that targeting the root-CSCs might be the ultimate method to kill all the bulk of cancer (Fig. 19.1).

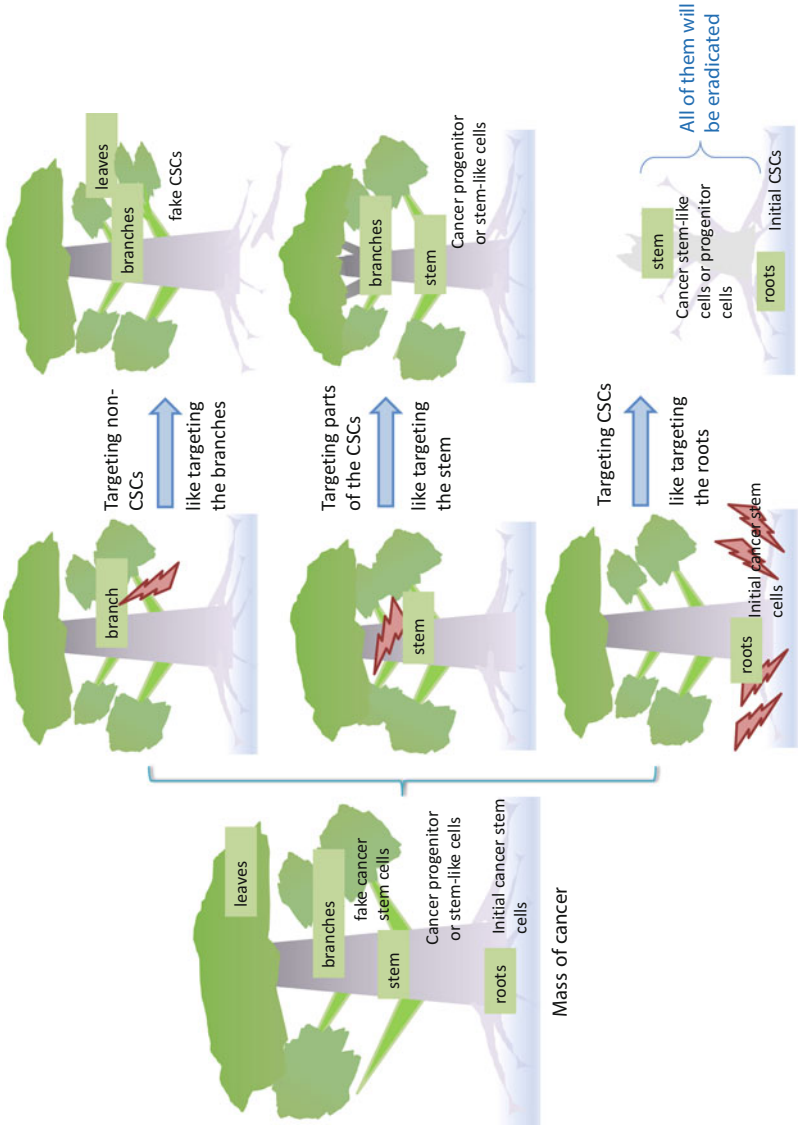

Fig. 19.1 Targeting cancer stem cells to eradicate cancer cells like targeting the roots of a tree

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