Current Human Cell Research and Applications
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Nariyoshi Shinomiya
Hiroaki Kataoka
Qian Xie *Editors*

Regulation of Signal Transduction in Human Cell Research

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Series Editors:

Nariyoshi Shinomiya
Department of Integrative Physiology and Bio-Nano Medicine
National Defense Medical College
Tokorozawa, Saitama, Japan

Hiroaki Kataoka
Department of Pathology
University of Miyazaki
Miyazaki, Japan

Yutaka Shimada
Department of Nanobio Drug Discovery
Kyoto University
Sakyo-ku, Kyoto, Japan

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Hiroaki Kataoka • Qian Xie
Editors

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Editors
Nariyoshi Shinomiya
Department of Integrative Physiology and Bio-Nano Medicine
National Defense Medical College
Tokorozawa
Saitama
Japan

Hiroaki Kataoka
Department of Pathology
University of Miyazaki
Miyazaki
Japan

Qian Xie
Department of Biomedical Sciences
Center of Excellence for Inflammation, Infectious Disease and Immunity,
Quillen College of Medicine,
East Tennessee State University,
Johnson
Tennessee
USA

ISSN 2522-073X ISSN 2522-0748 (electronic)
Current Human Cell Research and Applications
ISBN 978-981-10-7295-6 ISBN 978-981-10-7296-3 (eBook)
https://doi.org/10.1007/978-981-10-7296-3

Library of Congress Control Number: 2017964704

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The registered company is Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

Preface

Growth, survival, transformation, and metabolic activities at the cellular level are regulated by multiple intracellular and extracellular signaling transduction pathways. These pathways are the fundamental factors regulating human health. Human cells and animal models have been used widely to study the basic mechanisms of the signaling pathways and to develop therapeutic strategies toward human diseases. In this book, we review recent progress in elucidating these signaling pathways, their molecular regulation, and how their dysregulation may lead to cell death, metabolic and developmental diseases, and cancer. Highlighted topics include pathways mediated by microRNAs, TGF-β, Wnt, or HIF-1 and their relation to epithelial-mesenchymal transition, tumor microenvironment, and tissue development; DNA damage-mediated cellular response; G protein-coupled receptor signaling pathways; and the role of HGF/SF-MET signaling pathway in cancers and its activation mechanisms. The molecular design of small molecule inhibitors for targeting specific signaling pathways also is reviewed since it is becoming an important approach toward drug discovery.

While many books about signal transduction introduce how intracellular signals are regulated and transduced, this book is focused on the relationship between the regulation of signal transduction and clinical relevance. We also integrated how cell lines and animal models were used to facilitate the study of signaling pathways and preclinical applications. As such, this book is designed for medical students as well as researchers in the biomedical sciences.

Tokorozawa, Japan — Nariyoshi Shinomiya, M.D., Ph.D.
Miyazaki, Japan — Hiroaki Kataoka, M.D., Ph.D.
Johnson, TN, USA — Qian Xie, M.D., Ph.D.

September 5, 2017

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Chapter 1
Using Genetically Engineered Mouse Models to Study Wnt Signaling in Bone Development and Disease

Zhendong A. Zhong, Nicole J. Ethen, and Bart O. Williams

Abstract The skeleton supports the body structure and reserves calcium and other inorganic ions, and more roles played by bone are being proposed. The balance between bone formation (by osteoblasts and osteocytes) and bone resorption (by osteoclasts) controls postnatal bone homeostasis. For the past decade, a vast amount of evidence has shown that Wnt signaling plays a pivotal role in regulating this balance. Therefore, understanding how the Wnt signaling pathway regulates skeletal development and postnatal homeostasis is of great value for human skeletal health. We will review how genetically engineered mouse models (GEMMs) have been and are being used to uncover the mechanisms and etiology of bone diseases in the context of Wnt signaling.

Keywords Wnt signaling • Bone development • Transgenic mice • Conditional knock out • Cre-loxP • Tissue-specific promoter

Abbreviations

CKO	Conditional knockout
Fzd	Frizzled
GEMMs	Genetically engineered mouse models
GOF	Gain of function
KO	Full-body knockout
Lrp	Low-density lipoprotein-related receptor protein
LBM	Low bone mass
LEF	Lymphoid enhancer factor
LOF	Loss of function

Z.A. Zhong • N.J. Ethen • B.O. Williams (✉)
Program in Skeletal Disease and Tumor Microenvironment, Center for Cancer and Cell Biology, Van Andel Research Institute, Grand Rapids, MI, USA
e-mail: bart.williams@vai.org

© Springer Nature Singapore Pte Ltd. 2018

N. Shinomiya et al. (eds.), *Regulation of Signal Transduction in Human Cell Research*, Current Human Cell Research and Applications,
https://doi.org/10.1007/978-981-10-7296-3_1

MSC	Mesenchymal stem cell
M-CSF	Macrophage colony-stimulating factor
NA	Not applicable
OE	Overexpression
OMIM	Online Mendelian Inheritance in Man catalog
OPG	Osteoprotegerin
RANKL	Receptor activator of nuclear factor kappa-B ligand
TCF	T-cell factor

1.1 Wnt/β-Catenin Signaling

Wnt signaling is initiated by a conserved Wnt family of secreted glycolipoproteins, through β-catenin-dependent mechanisms (also known as canonical Wnt signaling) or in a β-catenin-independent manner (noncanonical Wnt signaling) [1, 2]. We will focus on the better characterized β-catenin-dependent Wnt signaling pathway, which plays fundamentally important roles in regulating cell fate decision, cell proliferation, and oncogenic events. In brief, without an upstream Wnt engaging the Wnt receptors, a "destruction complex" forms in the cytoplasm, where axin exists in the complex that includes the adenomatous polyposis coli (Apc) protein and the serine/threonine protein kinase GSK3 (glycogen synthase kinase 3). GSK3 phosphorylates β-catenin and targets it for ubiquitin-dependent degradation. When a Wnt engages a receptor complex (containing a member of the frizzled family of seven-transmembrane receptors and either Lrp5 or Lrp6), this induces the phosphorylation of the cytoplasmic tail of Lrp5/6, creating a binding site for axin. The recruitment of axin to the plasma membrane interferes with the ability of the destruction complex to recruit β-TrCP for ubiquitinylation and consequently blocks degradation of β-catenin. Then β-catenin accumulates in the cytoplasm and translocates into the nucleus (possibly due to other signaling events such as Rac1 activation), where it complexes with members of the LEF/TCF family of DNA-binding proteins to activate transcription of target genes (Fig. 1.1). This pathway is being intensively investigated, more components are being discovered, and more details about the pathway regulation are being unraveled.

1.2 Wnt/β-Catenin Signaling in Human Skeletal Diseases

The first line of evidence toward the role of Wnt signaling in bone was the finding that loss-of-function mutations in low-density lipoprotein-related receptor 5 (LRP5) are the cause of osteoporosis-pseudoglioma (OPPG) syndrome, a rare disease characterized by dramatic bone mass reduction and leukocoria. Further analyses showed that LRP5 was expressed in osteoblastic cells, which suggested that LRP5-mediated signaling in those cells might be responsible for this skeletal developmental defect [3]. Shortly after two independent groups reported LRP5 gain-of-function mutations that caused high bone mass of variable severity in two different families [4, 5].

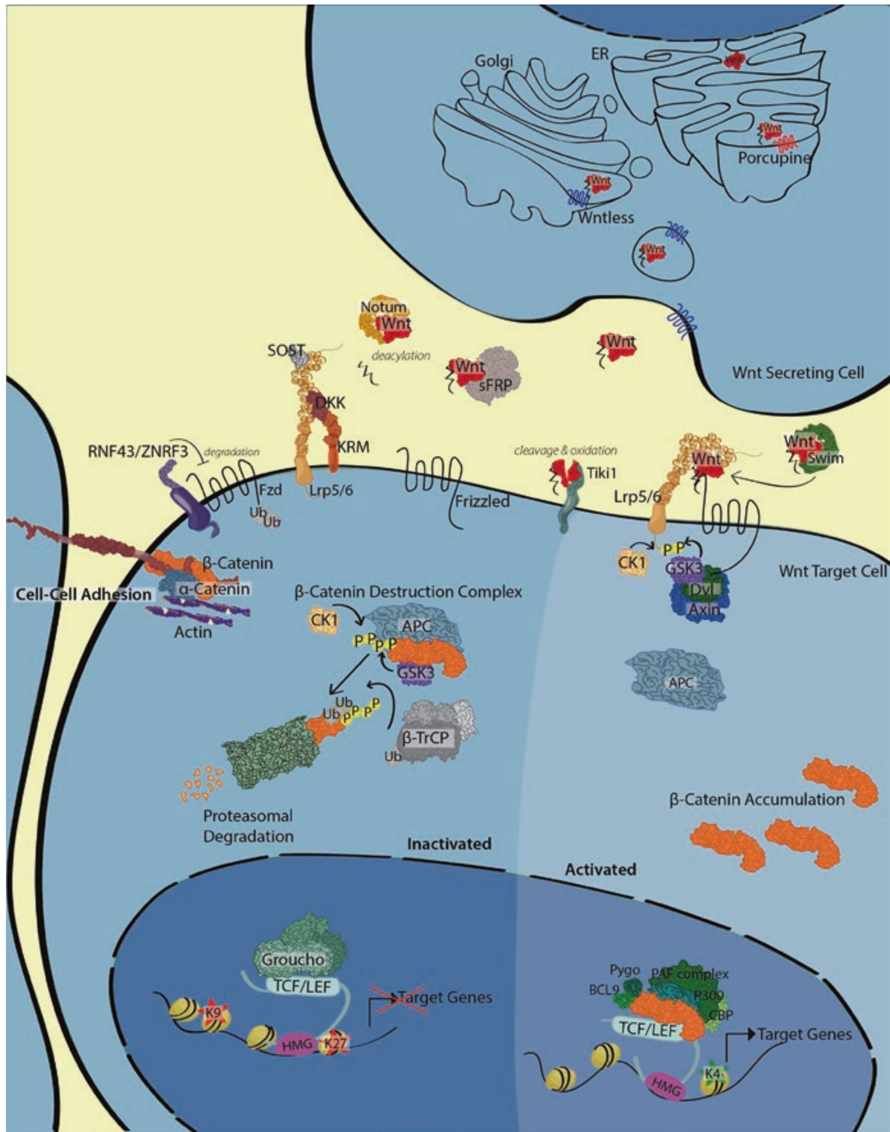

Fig. 1.1 Wnt/β-catenin (canonical) signaling pathway. Wnts are expressed and then lipid-modified by Porcupine in the endoplasmic reticulum (ER) compartment and are secreted out of the cell membrane with Wntless (Wls). Swim is proposed to be an extracellular carrier for Wnts to travel. In the absence of an upstream ligand, the receptor Fzd and coreceptor LRP5/6 are inactive. Cytoplasmic β-catenin will be recruited into a "destruction complex" consisting of axin, APC, CK1, and GSK3. This "destruction complex" facilitates the phosphorylation of β-catenin by GSK3 and subsequent ubiquitinylation (Ub) by β-TrCP, an E3 ligase. Because ubiquitinated β-catenin will be degraded in the proteasome, little β-catenin is accumulated in the cytosol or translocated into the nucleus, and the transcription repressor groucho occupies TCF/LEF. The signaling inactivity could be caused by unavailability of a coreceptor due to DKK1/SOST binding LRP5/6, inaccessibility of the receptor due to RNF43/ZNRF3 binding to Fzd for ubiquitinylation and degradation, or by the absence of active Wnts due to sFRP/Tiki/Notum and others binding to Wnts directly or enzymatically inactivating them.

Other missense mutations in LRP5 were associated with enhanced bone density in ten families and isolated patients [6]. Further, a loss-of-function mutation within an EGF-like domain of human LRP6, which is another important Wnt coreceptor, was identified to be associated with osteoporosis and metabolic syndrome in humans [7]. These findings implied an important role for LRP5 in regulating bone development and homeostasis, presumably through the Wnt/β-catenin signaling pathway. Other mutations in Wnt receptors related to human skeletal diseases are listed in Table 1.1.

Table 1.1 Skeletal phenotypes in mouse strains with germ line/global knockouts (KOs)

Gene		Phenotype(s) and related human skeletal disorders	Ref.
Wnt ligands	*Wnt1*	Swaying mice showed propensity to fractures and severe osteopenia due to defects in osteoblast activity. Wnt1 mutation recently found to be associated with osteogenesis imperfecta (OMIM: 166210)	[55, 56]
	Wnt3a	Homozygotes died by 12.5 dpc. Heterozygote had low bone mass (LBM) phenotype	[20, 57]
	Wnt3	Hypomorphic mutation (Vt) in Wnt3a caused exhibit vertebral abnormalities	[58]
	Wnt4	Homozygotes died at birth. Delayed chondrocyte maturation. Severe joint fusion with concomitant loss of Wnt9a	[59, 60]
	Wnt5a	Homozygotes died at birth. Shortened skeletal elements and loss of digits. Decreased hypertrophic chondrocytes and ossification that was most severe in distal bones. Heterozygotes showed LBM. Robinow syndrome (OMIM: 180700)	[57, 61, 62]
	Wnt7a	KO or inactivating mutation caused frequent loss of posterior digits and ectopic dorsal formation of sesamoid bones in paws. Al-Awadi/Raas-Rothschild/Schinzel phocomelia syndrome (OMIM:276820)	[21] [63]
	Wnt9a/14	Homozygotes died at birth. Decreased size and mineralization of appendicular bones. Ectopic cartilage nodules in cranial sutures and joints. Fusions of wrist and ankle bones	[60]
	Wnt9b	Hypomorphic mutation (clf1) in Wnt9b caused cleft palates. No skeletal assessments	[64]
	Wnt10b	Homozygotes were viable. LBM. Split-hand/split-foot malformation 6 (OMIM: 225300)	[65] [66]
	Wnt16	Homozygotes were viable. Reduced appendicular cortical bone mass and strength. Wnt16 KO mice had reduced cortical but not trabecular bone mass	[67, 68]

Table 1.1 (continued)

Gene		Phenotype(s) and related human skeletal disorders	Ref.
Wnt (co-) receptors	*Fzd2*	Homozygotes have reduced viability and cleft palate. Skeletal elements smaller but normal shape	[69]
	Fzd8	Homozygotes displayed osteopenia with normal bone formation and increased osteoclastogenesis	[70]
	Fzd9	LBM due to decreased bone formation. Williams-Beuren syndrome (OMIM: 194050)	[71]
	Lrp5	Different Lrp5 knockout models have been made by targeting different Lrp5 gene regions. Homozygotes were viable and showed LBM. Osteoporosis-pseudoglioma syndrome (OMIM: 259770)	[31, 72–75]
	Lrp6	Homozygotes died between E14.5 and birth. Numerous abnormalities including truncation of the axial skeleton, limb defects, and urogenital malformation. Heterozygotes had normal skeletogenesis and BMD but reduced BV/TV. Hypomorphic mutations (Cd, Rs) of Lrp6 caused skeletal defects, such as vertebral malformations, delayed ossification of the digits Coronary artery disease, autosomal dominant 2 accompanied by low bone mass (OMIM: 610947)	[75–79]
	Lrp4	Homozygous knockout died at birth. LOF mutations in Lrp4 (dan, mdig, mte, mitt) could cause brachydactyly and syndactyly on all limbs with duplications out of plane. Shortening and fusing of appendicular skeletal elements. Functional Lrp4 deficiency (Lrp4ECD) caused LBM. Cenani-Lenz syndactyly syndrome (OMIM: 212780); Sclerosteosis 2 (OMIM: 614305)	[80–83]
Other secretion factors	*Wls*	Homozygotes could not survive beyond E10.5 and showed defects in embryonic axis formation	[84]
	Dkk1	Homozygotes died at birth. Severe craniofacial malformation as well as fused and ectopic digits. Heterozygotes had no overt phenotype. However, BV/TV, mineral apposition rate, osteoblast surface, and mechanical resistance were increased. There was no change in osteoclasts	[85, 86]
	Dkk2	Homozygotes were viable. Osteopenia with major defects in mineralization rates. Increased osteoclast numbers, but no change in osteoblasts	[87]
	Sost	Homozygotes were viable. High bone mass similar to Lrp5-A214V (HBM mutation) with primary enhancement of cortical bone. Fracture healing was also enhanced. Sclerosteosis 1 (OMIM: 269500)	[88–90]
	Sfrp1	Homozygotes were viable. Skeletal elements and bone accrual normal but reduced age-related trabecular bone loss that was most pronounced in females	[91]
	Sfrp2	Homozygotes were viable. Shortened metacarpals/metatarsals and phalangeal bones with delayed ossification and a reduction of hypertrophic chondrocytes	[92]

(continued)

Table 1.1 (continued)

Gene		Phenotype(s) and related human skeletal disorders	Ref.
Intracellular factors	*Axin1*	Homozygotes died at E9.5. Heterozygotes had tail bifurcation and rib fusion. LOF mutations (Fu, Fu-kb) showed similar phenotypes with incomplete penetrance. Caudal duplication anomaly (OMIM: 607864)	[93] [94]
	Axin2	Homozygotes were viable but commonly developed craniosynostosis due to enhanced mineralization and ossification of the cranial sutures. Protection against age-related decreases in BMD and BV. Homozygotes of LOF mutation in axin2 (canopus, canp) died during gestation with shortened or doubled tails. Oligodontia-colorectal cancer syndrome (OMIM: 608615)	[95–98]
	Gsk3α	Viable mice were recovered with complete loss of Gsk3α in a heterozygous Gsk3β background. These mice were dwarfed with shorter limb bones and vertebrae. Chondrocyte zoning was normal however, there was metachromasia and decreased Col2a1 expression. Cultured chondrocytes had no change in β-catenin protein levels, cellular localization, or signaling ex vivo	[99]
	Gsk3β	Homozygotes died 24 h after birth. Cleft palate. Decreased ossification of the skull, ear bones, and cranial base. In contrast, heterozygous mice had increased cortical and trabecular bone mass, with no change in growth plate morphology. Bone formation rates were increased	[100–102]

Loss-of-function mutations of sclerostin (SOST), a Wnt antagonist, were found to be the causal events in sclerosteosis and Van Buchem's disease, which are both rare high-bone-mass genetic disorders [8]. SOST can bind Lrp5 and Lrp6 to suppress Wnt/β-catenin signaling [9]. LRP4 is an LDL receptor that closely resembles the extracellular domain of Lrp5/6, which was recently identified as a Wnt/β-catenin signaling antagonist presumably serving as a receptor of SOST [10, 11]. Two homozygous missense mutations in LRP4, which lead to LRP4 loss of function, were also identified as causes of sclerosteosis in human patients [12]. A key characteristic that makes the SOST gene particularly important and attractive for therapeutic targeting is that it is primarily expressed in osteocytes [9]. Thus, targeting SOST for osteoporosis treatment may cause fewer undesired side effects in non-skeletal tissues (reviewed in [13]). A number of pharmaceutical companies have generated different kinds of SOST inhibitors or neutralizing antibodies, including a small-molecule SOST inhibitor from OsteoGeneX that is in preclinical development [14]. Romosozumab, an anti-sclerostin antibody from Amgen, was reported to increase bone mineral density in healthy postmenopausal women, presumably and mechanistically due to both enhanced bone formation and repressed bone resorption [15]. Blosozumab, another SOST antibody developed by Eli Lilly, appears to have similar effects [16].

Alterations in Wnt signaling identified in human skeletal diseases indicate a pivotal role for this pathway in bone. With more sophisticated screenings, the revolu-

tion of sequencing technology and an improved understanding of the Wnt signaling pathway, ever more genetic changes within Wnt signaling components, are being identified as related to or causal to human bone mineral density or specific skeletal diseases (Table 1.1). Using genetically engineered animal models, we can not only confirm the importance of these genes in bone development and homeostasis but also explore potential therapeutic interventions targeting the Wnt signaling pathway.

1.3 Transgenic and Germ Line Knockout Models

The first so-called "transgenic" mice that facilitated the expression of exogenous proteins in specific tissues were created by pronuclear injection of appropriately designed segments of DNA into one-cell embryos shortly after fertilization (Fig. 1.2, top left panel). Typically, the DNA segment would contain promoter sequences that drove expression of an included cDNA with the necessary polyadenylation signal in a tissue of interest [17]. These models provided important insights into biological functions for many decades but were limited by the fact that the insertion of the DNA segment is a somewhat random process, so the expression level of the transgene may be heavily influenced by the site of insertion.

The next development was the ability to create mice carrying targeting gene inactivation in the germ line. The techniques that facilitated these approaches were first described in the late 1980s, and their importance in many areas of biomedical research is best illustrated by the fact that the pioneering investigators were awarded the Nobel Prize in Physiology and Medicine in 2007 [18]. These revolutionary techniques exploit the ability to identify clones of mouse embryonic stem cells in which a specific recombination event has occurred. The creation of these genetically engineered mouse models (GEMMs) could take many months, but the biological insights from the resulting GEMMs made these investments of time and resources worthwhile.

Compared with overexpressing a specific gene in a transgenic mouse model, knockout mouse models provide valuable clues about what genes can do in physiological settings. Since mice share many genes with humans, observing the characteristics of a knockout mouse model allows researchers to better understand how similar genes in humans may cause or contribute to diseases. The initial gene function characterization is usually performed on germ line knockout mice. A historical footnote is that one of the first genes chosen for targeted inactivation in the mouse germ line was *Int1* (later called *Wnt1*) [19]. The involvement of the Wnt/β-catenin signaling pathway in skeletal development was first observed in Wnt3a and Wnt7a knockout embryos: Wnt3a-targeted embryos had axial defects and Wnt7a-targeted embryos showed limb-development defects [20, 21]. Although germ line knockouts of important genes would cause embryonic death or serious developmental defects, it is still the most efficient and convenient way

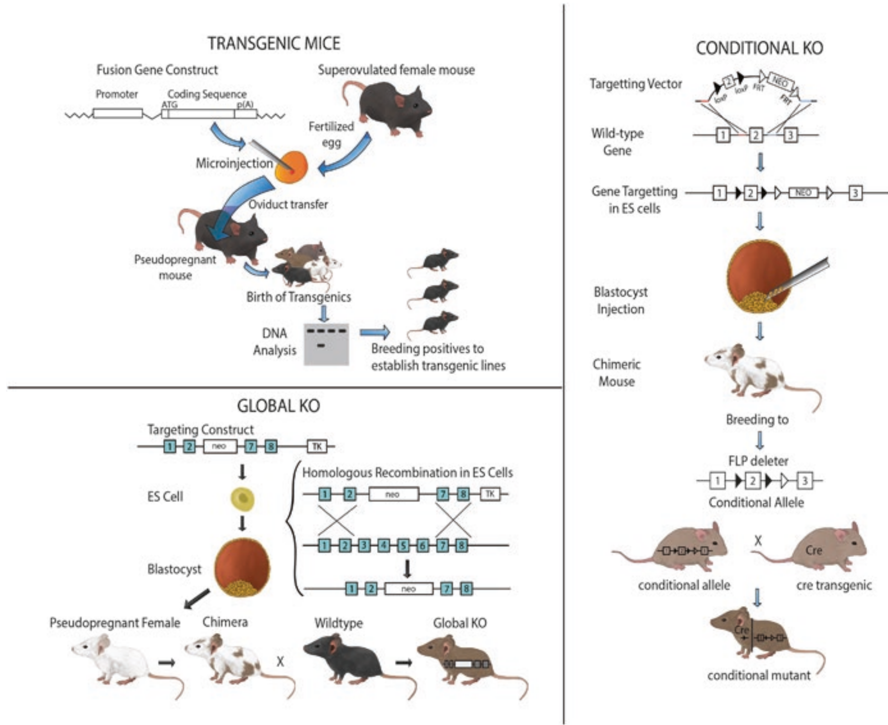

Fig. 1.2 Knockout and transgenic mouse models. *Transgenic mice*: Transgenic mice that facilitate the expression of exogenous proteins in specific tissues are created by pronuclear injection of appropriately designed segments of DNA ("fusion gene") into one-cell embryos shortly after fertilization. The fusion gene contains promoter sequences that drive expression of an included cDNA with the necessary polyadenylation signal (p(A)) in a tissue of interest. Since the insertion of the DNA segment is a somewhat random process, the offspring are analyzed for transgene integration, and then the positive founders are used to establish transgenic lines with stable expression of the transgene. *Global knockout*: A targeting construct contains part of the gene for homologous recombination (exon 1/2 and 7/8), a selectable marker (TK), and a reporter gene (neo, also another selectable marker). This construct is injected into mouse embryonic stem cells in culture, and then the cell clones with correct recombination will be used to contribute to the mouse's tissue via blastocyst injection. The resulting chimeric mice, where the modified cells make up the reproductive organs, are selected for via breeding with wild-type mice. *Conditional knockout*: A conditional targeting vector typically contains a part of critical exons flanked by LoxP sites and a selective marker ("Neo") flanked by FRT sites. Upon gene targeting in ES cells, the vector can change an endogenous gene through homologous recombination and subsequently be screened by treatment with antibiotics (neo) or with PCR. After blastocyst injection, chimeric mice are identified with PCR and further crossed with a FLP deleter mouse to remove the neo gene, subsequently generating an inheritable conditional allele. When bred with a Cre mouse, the "floxed" mouse carrying the conditional allele will permanently remove the floxed exon(s) in the Cre-expressing tissues

to study a novel gene. These approaches generated significant insights into how genes from the Wnt signaling pathway regulate skeletal development (Table 1.1). However their embryonic lethal character often precluded the detailed characterization of bone homeostasis.

1.4 Bone-Specific Conditional Knockout Models

The advances in Cre-lox recombination systems to create enhanced GEMMs have helped to study gene function in specific tissues or cell types. The Cre-lox system was identified in bacteria [22]. A loxP (locus of X-over P1) site is a 34-base-pair consensus sequence containing a core domain of 8 base pairs flanked on each side by a 13-base-pair palindromic sequence [23]. The Cre recombinase is 38 kDa and catalyzes recombination between two of its sequence recognition (loxP) sites, resulting in the elimination of sequences flanked by the loxP sites. This led to the development of numerous mouse strains in which essential portions on a gene are flanked by loxP sites (so-called "floxed" strains). If the Cre gene is expressed in a cell type via the use of well-characterized tissue-specific promoter, Cre-mediated recombination leads to loss of gene function in that particular cell type (Fig. 1.2, right panel). Therefore, the specificity of Cre expression controls where the conditional knockout will occur. To regulate Cre expression spatially and temporally, the Cre recombinase gene is inserted into the genome under the transcriptional control of one of the promoters that would be active in a particular cell type and at a particular stage during skeletal development. Several Cre strains have been created via pronuclear injection followed by random integration of the expression plasmid, while others have been developed using homologous recombination in mouse embryonic stem cells to target Cre expression to occur from endogenous promoters [Fig. 1.3].

1.4.1 Bone-Specific Promoters

In order to accurately evaluate the role of a gene in a particular tissue/cell type using the Cre/lox system, the promoter specificity and penetrance (the percentage of target cells that express Cre) are two major considerations. To characterize the Cre activity, a reporter mouse model that harbors a loxP-flanked DNA STOP sequence in front of a reporter gene (LacZ or EGFP) in the genome is widely used (Fig. 1.4). Upon being crossed with a Cre strain, the STOP codon would be removed to activate the downstream reporter gene expression in those tissues/cells where Cre activity is present. X-gal (an analog of lactose) staining is widely used to locate which cells express a LacZ reporter gene that produces β-galactosidase enzyme [24]. More recently, scientists have developed more sensitive reporter models using similar strategies, such as the mT/mG model with dual-fluorescent protein labeling [25]. Using the mT/mG reporter mouse

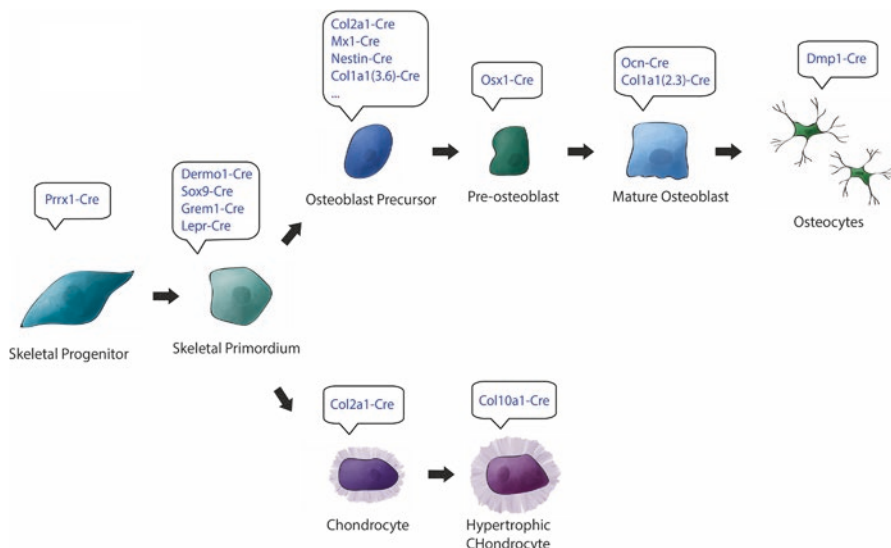

Fig. 1.3 Osteochondral cell differentiation and bone-specific promoters. Chondrocytes, osteoblasts, and some other cell types likely originate from common skeletal progenitor cells. Chondrocytes and osteoblasts express different sets of genes throughout their maturations. The corresponding bone-specific promoters target osteoblasts and chondrocytes at different maturation stages

line, our laboratory was able to detect earlier osteocalcin promoter activity in osteoblasts than could be detected with the LacZ system [26, 27]. Such reporter systems are important in assessing Cre activity, because many promoters have extraskeletal expression patterns that need to be carefully considered in interpreting phenotypes. By comparing the phenotypes of conditional knockout animals with multiple and independent Cre strains, we can often better evaluate and compare the roles of a specific gene at various stages of a lineage or in closely related cell types.

We next will review conditional knockouts that happen in three of the major cell types within the skeleton: osteoblast, chondrocyte, and osteoclast. These three cell types regulate bone development and bone remodeling, and they are involved in the pathogenesis of skeletal diseases such as osteoporosis and osteoarthritis. The interplay between osteoblasts and osteoclasts regulates the balance between bone formation and bone resorption to maintain skeletal homeostasis [Fig. 1.5]. Some promoters are active in the precursors to both osteoblasts and chondrocytes (such as Prrx1-Cre and Dermo1-Cre). More profound phenotypes may be observed in conditional knockout models with Cre drivers that are expressed earlier and in more cell types. GEMMS have demonstrated that Wnt signaling is important for the commitment of mesenchymal stem cells (MSCs) to the osteoblast lineage. However constitutive activation of Wnt signaling may prevent osteoblastic terminal differentiation [28, 29].

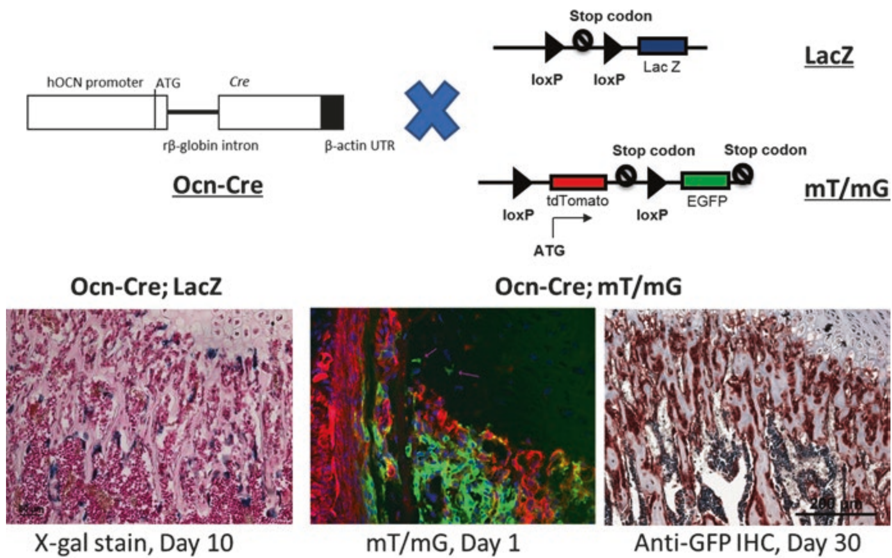

Fig. 1.4 Cre reporter strains. A "LacZ" cassette contains a LacZ gene preceded with a STOP codon. The STOP codon will be removed upon exposure to a Cre recombinase to allow LacZ expression. Similarly, an "mT/mG" cassette contains a tdTomato gene (red fluorescent) preceding a GFP gene, so that it expresses tdTomato until a Cre removes the "tdTomato-STOP" cassette and activates the GFP expression. These transgene cassettes are inserted into ROSA26 genome locus to facilitate ubiquitous expression. Upon being crossed with a tissue-specific Cre driver (the osteocalcin promoter, for example), Cre activity can be detected by X-gal staining, direct fluorescence microscopy examination, or anti-GFP immunohistochemistry

1.4.2 Osteoblast-Specific Knockouts

Osteoblasts are specialized, differentiated products of mesenchymal stem cells (MSCs), and they are terminally differentiated into osteocytes once they are imbedded in bone matrix. Osteoblasts synthesize large amounts of cross-linked collagen and smaller amounts of several other proteins, including osteocalcin and osteopontin, to allow for the formation of the organic matrix of the bone. The osteoblastic lineage is defined at different stages based on biological behavior and transcription markers, and more cell stage-specific markers are being discovered. Using the corresponding promoters, one can delete a gene at a specific maturation stage. So far, most evidence suggests that Wnt/β-catenin signaling in osteoblasts inhibits bone resorption or augments bone formation (Table 1.2). Our group and others have proposed that Wnt/β-catenin signaling in osteoblasts could regulate osteoclast activity by regulating osteoprotegerin (OPG) expression [28, 30]. OPG inhibits osteoclastogenesis by binding to RANKL and preventing it from interacting with RANK on osteoclast precursors, and OPG protects the skeleton from excessive bone

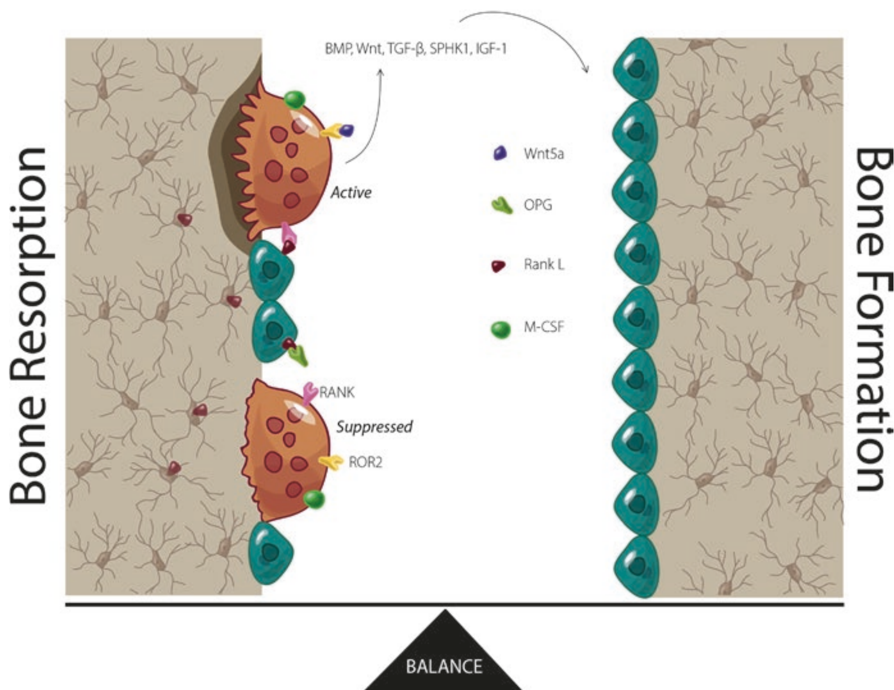

Fig. 1.5 Bone formation and bone resorption. Bone homeostasis is regulated by the balance between osteoblast-mediated bone formation and osteoclast-mediated bone resorption. The activities of osteoblasts and osteoclasts are coupled at least partially through secreted factors. Osteoclasts or osteoclast activity stimulates osteoblast differentiation or homing to bone resorption site by secreting BMP, Wnt, TGF-β, IGF-1, SPHK1, and other factors. RANKL, M-CSF, and Wnt5a are key factors for osteoclast differentiation and maturation, which are expressed by osteoblasts. Another secreted factor from osteoblasts, OPG, acts as RANKL decoy receptor and inhibits RANKL-RANK interaction, therefore impairing osteoclast maturation

Table 1.2 Skeletal phenotypes in mouse strains with conditional knockouts (cKOs)

Gene	Cre line (targeted tissue)	Phenotype(s)	Ref.
Lrp5	CMV-Cre (Germ line)	Similar phenotype to the other Lrp5-KO mice	[103]
	Dermo1-Cre	Normal skeletogenesis. No other skeletal assessments	[103]
	(2.3kb)Col1a1-Cre or Villin-Cre (intestines)	No change in bone mass with Col1a1-Cre, but reduced bone mass with Villin-Cre	[31]
	Dmp1-Cre (osteocytes) or Villin-Cre	No change in bone mass with Villin-Cre, but reduced bone mass with Dmp1-Cre	[34]

Table 1.2 (continued)

Gene	Cre line (targeted tissue)	Phenotype(s)	Ref.
β-Catenin	Col2a1-Cre) or Dermo1-Cre	Homozygotes died shortly after birth. Skeletal defects from Col2a1-Cre crossings include shortened limbs, loss of tarsal synovial joints leading to bone fusion and domed skulls. Dermo1-Cre produced a more severe phenotype	[104]
	Brn4-Cre (neural tube and hind limbs)	Malformation of hind limbs, including truncation or absence of tibia, fibula, and digits	[105]
	(2.3kb) Col1a1-Cre	Homozygotes were viable. Decreased bone mass. Increased osteoclast activity. No change in osteoblasts.	[30]
	Ocn-Cre	Homozygotes died within 1 month of birth with decreased bone mass. Increased osteoclast activity	[28]
	Prrx1-Cre	Mice died at birth. Appendicular bones were shortened, partially fused, with loss of distal structures and mineralization. Growth plate chondrocyte zoning was normal, but bone collars were absent	[40]
	Dermo1-Cre	Homozygotes exhibited an inhibition of commitment to the osteoblast lineage with severe defects in skeletogenesis	[40, 106]
	Osx1-Cre-TetOff	Severe skeletal defects due to a lack of fully differentiated osteoblasts, which resulted in no mineralization or ossification of bone	[107]
	PPARγ-tTA (osteoclast progenitors) + TRE-Cre	Heterozygotes were osteoporotic with decreased trabecular and cortical bone mass. Increased osteoclast number, and surface area with no change in osteoblasts. Interestingly, homozygotes were osteopetrotic, similar to, but not as severe as the β-catenin GOF allele	[42]
	Dmp1-Cre	Homozygotes were viable. Growth retardation and early lethality at 3-5 months. Early onset cortical and cancellous bone loss in both the appendicular and axial skeleton. Osteoclast number and activity were elevated, with no change in osteoblasts	[108]
	Col2a1-Cre-ERT2 (chondro-osteo progenitors-inducible)	Reduced hypertrophic chondrocyte zone, disorganization of prehypertrophic chondrocytes, and a reduction of the primary ossification center. By E18.5, there was a failure of vascularization of the cartilage lacunae	[109]
	Osx1-Cre-ERT2 (tamoxifen inducible)	Tamoxifen induced recombination was performed in 2-month-old mice. Osteopenia developed by day 21 characterized by reduced Tb.N and increased Tb.Sp, but no change in trabecular thickness. Decreased cortical bone by day 49. Osteoclast numbers and activity were elevated at day 21 and beyond. Serum PINP levels and osteoblast numbers were decreased at day 8 but elevated in the subsequent time points	[110]

(continued)

Table 1.2 (continued)

Gene	Cre line (targeted tissue)	Phenotype(s)	Ref.
Porcn	Sox2-Cre (epiblast)	Heterozygous females were viable. Shortened or absent limbs and digits with variable severity. No skeletal or bone specific Cre model assessments. Focal dermal hypoplasia (OMIM: 305600)	[111]
	Msx2-Cre (hind limb ectoderm) or Prrx1-Cre	Shortened hind limbs and syndactyly of digits (Msx2-Cre). Shortened limbs and loss of digits, but no syndactyly (Prrx1-Cre)	[111]
	Chimeras	FRT-Neo cassette created a hypomorphic allele in chimeras. 9/17 resulting male and female chimeras had absent, fused, or shortened digits on limbs and vertebral abnormalities	[112]
	EIIa-Cre or Hprt-Cre (germ line)	Viable male and female mice in low yield with abnormal hair follicle development (Ella-Cre). Hprt-Cre resulted in heterozygous females in low yield. Frequent axial truncations and neural tube malformations in embryos. No skeletal or bone specific Cre model assessments	[112]
	Prrx1-Cre	Similar to the Barrott et al. mouse but with syndactyly of soft tissue and no loss of digits.	[112]
Wls	Ocn-Cre	Progressive decreases in BMD, trabecular, and calvarial bone, as well as cartilage starting at 20 days of age. <20% of mice survived over 2 months. Massive decreases in BV/TV and cortical bone by 7 weeks. Decreased serum osteocalcin. Increased TRAP staining	[47]
	Prrx1-Cre	Homozygotes died at weaning. Limbs were hypoplastic, shortened, with truncated autopods. Ossification, chondrocyte hypertrophy, and osteogenesis were also impaired	[113]
	Msx2-Cre	Truncated autopods in all limbs, but shortened zeugopods only in hind limbs. Defective suture fusion and ossification of the skull	[113]
	Dermo1-Cre	Reduced mineralization, malformation, and/or absence of skeletal elements during skeletogenesis. Loss of hypertrophic chondrocytes	[114]
	(2.3kb)Col1a1-Cre or Osx-Cre	No defects in skeletogenesis or bone mineralization at embryonic stage. Adult bone phenotype was similar to Ocn-Cre-driven wls cKO mice	[114, 115]
	Col2a1-Cre	Shortened long bones, loss of bone collar formation, decreased bone mineralization, and reduced chondrocyte maturation	[114]

resorption by osteoclasts. Some evidence suggested that β-catenin and cofactors could directly bind to the promoter region of the OPG gene and activate its transcription [30]. Currently, several OPG analogs or RANKL antibodies are being evaluated for improving bone homeostasis in clinical trials.

Another model proposes that Lrp5, a Wnt coreceptor, controls osteoblast differentiation by regulating the amount of serotonin that is secreted from intestinal enterochromaffin cells. The varying serotonin levels that bind to their receptors on osteoblasts then regulate differentiation and bone formation [31–33]. However, a great deal of evidence supports the model described above, in which the activity of the Wnt signaling pathway within the osteoblast is regulated to control skeletal development and homeostasis [33–35]. The reasons for the discrepancies between these two models remain unclear.

1.4.3 Chondrocyte-Specific Knockouts

Both chondrocytes and osteoblasts are derived from mesenchymal stem cells (MSCs). Chondrocytes in the growth plate will further differentiate into hypertrophic chondrocytes, which support endochondral ossification. Although we do not focus on osteoarthritis in this review, articular chondrocytes play important roles in maintaining articular cartilage and joint function. The dysregulation of articular cartilage by Wnt/β-catenin signaling in chondrocytes is directly connected to the process of cartilage degeneration in osteoarthritis [36, 37, 41]. It's important to note that a recent fate-mapping study convincingly showed that all tested promoters proposed to have "chondrocyte-specific" activity (including Acan, Col2, and Sox9, all of which are important transcription factors during chondrogenesis) could be chased into osteoblastic and other lineages, which means deleting genes in chondrocytes may also delete genes in the osteoblastic lineage [38]. Higher Wnt/β-catenin signaling in osteoprogenitor cells favors osteogenesis over chondrogenesis, while lower Wnt/β-catenin signaling seems to do the opposite [35, 39, 40]. In committed chondrocytes, Wnt/β-catenin signaling actually promotes chondrocyte proliferation and maturation [29, 37, 41], so it is important to interpret the phenotypes of conditional knockout animals based on the Cre driver's specificity and timing.

1.4.4 Osteoclast-Specific Knockouts

Conditionally activating β-catenin in any stage of the osteoclast lineage could cause osteopetrosis with suppressed bone resorption. However, inactivation of β-catenin in osteoclast precursors (driven by PPARγ or Tie2 promoter) showed dose-dependent effects: β-catenin heterozygosity enhanced osteoclast differentiation, but β-catenin deletion suppressed osteoclast precursor proliferation. Deletion of

β-catenin in more committed stages of osteoclast differentiation (driven by the LyzM or Ctsk promoter) enhanced osteoclast differentiation and bone resorption in vivo [42]. More recent work showed that Wnt3a could inhibit osteoclastogenesis by inactivating NFATc1 in osteoclast progenitors through the β-catenin-independent and Lrp5-/6-dependent signaling pathway, while RANK-Cre-driven Lrp5/6 deletion also suppressed osteoclast precursor proliferation [43].

1.5 Gain-of-Function Mutations and Transgenic Models

In some cases, mutations can cause gene products to become constitutively active. For example, single amino acid mutations (such as A214V or G171V) in the *LRP5* gene, which are found in high-bone-mass human patients, can reduce the ability of the endogenous inhibitor SOST to bind both Lrp5 and Lrp6 and thus increase bone formation caused by mechanical load [4, 5, 34]. Another example is the deletion of exon 3 in the β-catenin gene, causing stabilized β-catenin protein that cannot be phosphorylated, so that β-catenin is constitutively activated and causes profound effects on target tissues (Table 1.3).

As discussed above, transgenic models often contain genes of interest that are driven by an endogenous promoter (by targeted insertion at a selected locus) or an exogenous promoter (inserted into the genome by random or homologous recombination). Although these caveats require some caution in interpreting the resulting phenotypes, the creation of GEMMs remains among the most powerful methods for studying mammalian gene function and regulation, because it can be carried out on the whole organism or in a tissue-specific manner. For example, to understand the novel SOST gene's function in bone, the initial study was performed with a transgenic mouse model with osteoblast-specific expression of mutant SOST (driven by the mouse osteocalcin promoter, OG2) [44], which has been shown to be associated with sclerosteosis [8, 45]. Please refer to Table 1.4 for more examples of transgenic mouse models that manipulate Wnt/β-catenin signaling in the skeleton.

1.6 Detection of Wnt/β-Catenin Signaling Change in Genetically Modified Animals

After a gene linked to regulation of the Wnt pathway has been genetically modified, confirmation that it affects Wnt signaling in target cells is necessary before characterizing the skeletal phenotypes. A reporter mouse strain (BAT-GAL) that expresses β-galactosidase driven by a promoter with multimerized LEF/TCF-binding sites is a sensitive tool to detect Wnt/β-catenin signaling changes. In the presence of activated β-catenin, β-galactosidase expression will be activated and can be detected by its substrate, X-gal [46] (Fig. 1.6). Several other models can detect Wnt signaling

Table 1.3 Skeletal phenotypes in mouse strains with gain-of-function mutations

Gene	Mutations	Cre line (targeted tissue)	Phenotype(s)	Ref.
Lrp5	One amino acid change increases signaling capacity			
	A213V (mimics human Lrp5-A214V)	Germ line	Increased cortical bone mass, BV/TV, Tb.N, and Tb size with a decrease in Tb Sp. Increased mechanical resistance. Endosteal hyperostosis, autosomal dominant (OMIM: 144750)	[34, 90]
	A213V (mimics human Lrp5-A214V)	Dmp1-Cre , Prrx1-Cre, or Villin-Cre (intestine)	Global increases in bone mass with Dmp1-Cre that mimicked global Lrp5-A214V expression. Increased bone mass in limbs only with Prrx1-Cre. No change in bone mass with Villin-Cre. Osteosclerosis (OMIM: 144750)	[34]
	G170V (mimics human Lrp5-G171V)	Germ line	Global increases in cortical bone mass, BV/TV, Tb.N, and trabecular thickness with a decrease in Tb.Sp. Greater enhancement of endosteal bone formation during development and in response to load. Osteopetrosis (OMIM: 607634)	[34, 90]
	G170V (mimics human Lrp5-G171V)	Dmp1-Cre, Prrx1-Cre, (2.3kb) Col1a1-Cre, or Villin-Cre (intestine)	Global increases in bone mass with Dmp1-Cre that mimicked global Lrp5-G171V expression. Increased bone mass in limbs only with Prrx1-Cre. Villin-Cre showed no effect on bone mass in one study (Cui et al., 2011), but resulted in global bone mass enhancement in another (Yadav et al., 2008). Additionally, the latter group showed no enhancement of bone mass when Col1a1-Cre was used to activate Lrp5-G171V in bone. High bone mass (OMIM: 601884)	[31, 34]

(continued)

Table 1.3 (continued)

Gene	Mutations	Cre line (targeted tissue)	Phenotype(s)	Ref.
β-Catenin	Exon 3 deletion causes constitutive β-catenin activation			
		Col2a1-Cre or Dermo1-Cre	Heterozygotes died around E18 with severe and generalized chondrodysplasia. Reduced endochondral bone size	[116]
		Brn4-Cre (neural tube and hind limbs)	Enlarged hind limb buds during development	[105]
		(2.3kb) Col1a1-Cre	Heterozygotes failed to thrive and died shortly after weaning. Skeletal assessments revealed greatly increased bone mass and cartilaginous deposits in long bones and vertebrae. Osteoblast numbers were normal, but Col1a1 expression was increased. Osteoclasts were decreased	[30]
		Prrx1-Cre	Heterozygotes died at birth with loss of limb and the skull bones	[40]
		Osx1-Cre-TetOff (Tetracycline inhibitable)	Heterozygotes died at birth. Shortened long bones with premature ossification and mineralization. Loss of hypertrophic chondrocytes	[107]
		Col2a1-Cre-ERT2 (tamoxifen inducible)	Tamoxifen-induced recombination at 3 or 6 months resulted in a loss of articular cartilage 2 months later	[37]
		Col2a1-Cre-ERT2 (tamoxifen inducible)	Tamoxifen-induced recombination at E13.5 followed by skeletal assessments at several embryonic time points. Disordered chondrocyte zoning. Increased thickness of the perichondrial bone collars in limbs. TRAP staining showed no change in osteoclast activity	[109]
		Axin2-rtTA (Wnt responsive cells) + TRE-Cre [functions as a doxycycline inducible axin2-Cre]	Expansion and increased ossification of cranial sutures. Fully mature osteoblast differentiation was inhibited. No additional skeletal assessments	[117]
		PPARγ-tTA (osteoclast progenitors) + TRE-Cre [functions as a doxycycline inhibitable PPARγ-Cre]	Severe osteopetrosis with a 27-fold increase in the BV/TV ratio. Trabecular bone and cortical bone mass were both increased. Histomorphometry revealed large decreases in osteoclast number and surface area, with no effect on osteoblasts. The osteopetrotic phenotype was already evident in 15-day-old pups. Osteopetrosis could also be induced by removing Dox in adult mice	[42]

Table 1.4 Skeletal phenotypes in transgenic mouse strains

Gene	Transgene	Target tissue	Phenotype(s)	Ref.
Wnt4	R26-Flox-Neo-Wnt4	Col2a1-Cre (osteochondral progenitors)	Dwarfism with increased hypertrophic chondrocytes in growth plates of long bones. Normal BMD	[118]
Wnt5a	Col2a-Wnt5a	Osteochondral progenitors	Shortened skeletal elements and delayed ossification. Increased growth plate cartilage with a large zone of undifferentiated chondrocytes with a low index of proliferation	[62]
Wnt8a	β-Actin-Wnt8a (*Gallus gallus*)	Germ line	Axis duplication during early embryo development	[119]
Wnt9a/14	Col2a-Wnt9a	Osteochondral progenitors	Homozygotes died by 16.5 dpc. Decreased cartilage	[104]
Wnt10b	FABP4-Wnt10b	Adipocytes (adipose tissue and bone marrow)	Mice were viable. Increased Tb.N and decreased Tb.Sp. Increased mechanical resistance. Decreased rate of age-related and ovariectomy-induced bone loss	[65]
Wnt5b	Col2a-Wnt5b	Osteochondral progenitors	Similar phenotype as Col2a-Wnt5a mice, but the undifferentiated chondrocytes were highly proliferative	[62]
Dkk1	Col2a1-Dkk1, Col10a1-Dkk1, or Tie2-Dkk1	Osteochondral progenitors, hypertrophic chondrocytes, or endothelial cells	There was no change in the overall pattern of cartilage or bone development in chondrocyte overexpression of Dkk1 Overexpression in endothelial cells resulted in smaller skeletal elements, abnormal hypertrophic chondrocytes, reduced Tb size but an increase in Tb.N. TRAP staining and osteoclast numbers at the hypertrophic chondrocyte-trabecular bone interface were decreased. The mineral apposition rate was unaffected	[120, 121]

(continued)

Table 1.4 (continued)

Gene	Transgene	Target tissue	Phenotype(s)	Ref.
Dkk1	(2.3kb) Col1a1-Dkk1	Mature osteoblasts	Reduced BMD and trabecular BV. Osteoblast surface area and number were reduced. Osteoclasts were normal	[122]
Dkk2	Col2a1-Dkk2, Col10a1-Dkk2, or Tie2-Dkk2	Osteochondral progenitors, hypertrophic chondrocytes, or endothelial cells	There was no change in the overall pattern of cartilage or bone development in chondrocyte or endothelial overexpression of Dkk2	[120]
SOST	Ocn-SOST	Osteoblasts and osteocytes	Mice were viable but osteopenic with disorganized bone architecture, thin cortices, reduced trabecular bone, and chondrodysplasia. Decreased osteoblast surface and reduced bone formation rate, with no change in resorption markers	[44]
Sfrp4	SAP-Sfrp4	Various	Decreased rate of bone acquisition from 5-15 weeks after birth. Decreased BV/TV and Tb size	[123]
Sfrp4	Transgene (2.3kb) Col1a1-Sfrp4	Mature osteoblasts	Similar phenotype as the SAP-Sfrp4 transgenic mice	[124]
Tcf1 (Tcf7)	Col2a1-Tcf7 Δβ-catenin-binding domain	Osteochondral progenitors	Reduced skeletal element size, endochondral ossification, chondrocyte maturation, and proliferation	[125]
Lef1	(2.3kb) Col1a1-Lef1ΔN isoform	Mature osteoblasts	Increased trabecular BV and osteoblast activity, but no change in osteoblast number. Osteoclasts were normal	[126]
Sfrp4	(2.3kb) Col1a1-Sfrp4	Mature osteoblasts	Osteoblast-targeted expression of Sfrp4 in mice results in low bone mass	[124]

activity in cells and have been widely used with great success. In addition, immunohistochemical analysis of β-catenin or downstream targets is also routinely performed to detect the signaling changes [47](Fig. 1.6).

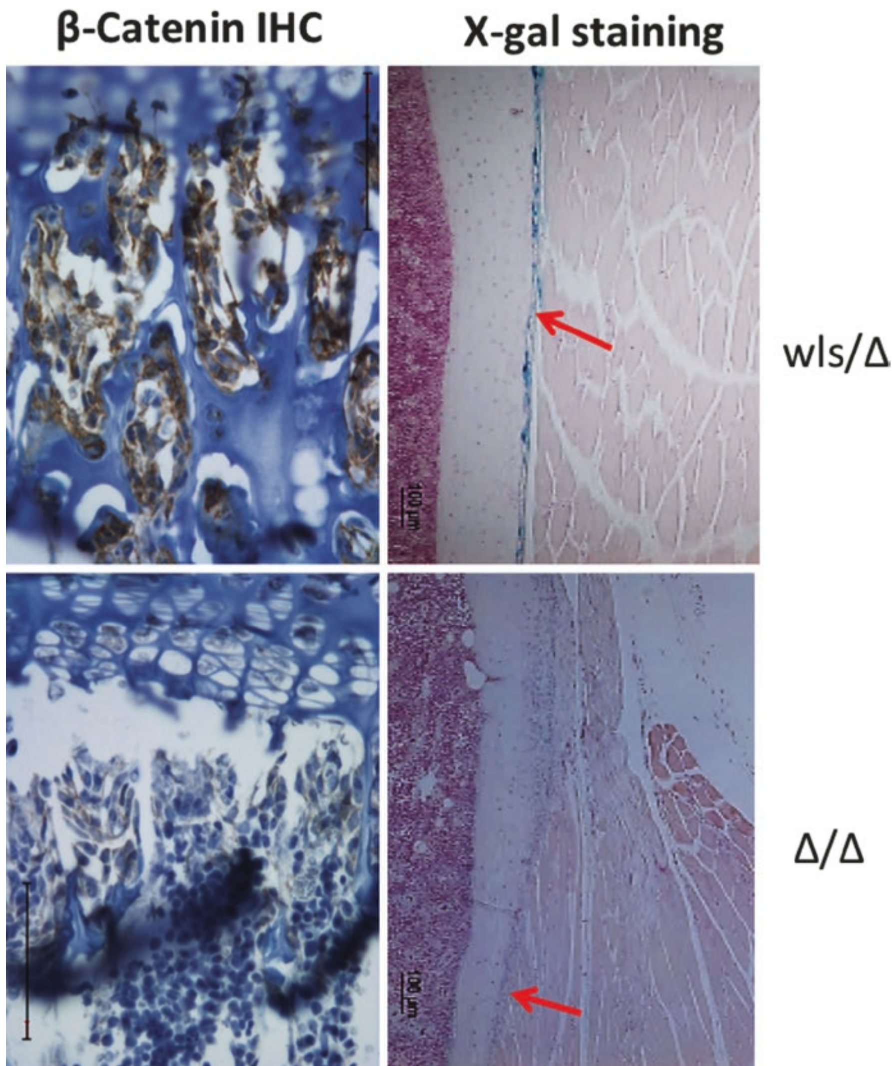

Fig. 1.6 Wnt/β-catenin signaling detection. An example of a conditional mouse model with osteoblast-specific Wntless (wls) knockout is shown. Anti-β-catenin IHC on distal femur is shown to indicate lower β-catenin in the mutant trabecular bone. BAT-GAL transgenic mice (also called β-catenin/TCF/LEF reporter transgenic mice) can express β-galactosidase in the presence of activated β-catenin, so X-gal staining (blue) can identify those cells with activated β-catenin signaling

1.7 Conclusion

A "pipeline" project that aims to knock out every gene in the genome individually has predicted that about 10% of all genes can affect bone strength in one way or another [48]. Further evidence for this assessment was recently provided by Lexicon Genetics, which reported the results of their efforts to screen large numbers of GEMMs to identify new regulators of skeletal development and homeostasis. The fact that the Notum gene, recently linked to direct regulation of the Wnt pathway in other systems [49, 50], was identified as a novel, targetable regulator of bone homeostasis speaks to the power of these types of approaches [51].

We hope that this review has provided a convincing argument for the critical role that GEMMs have played in our understanding of how Wnt signaling regulates skeletal development and homeostasis. While the last three decades of using GEMMs have led to many exciting discoveries, we predict that these insights will grow exponentially in the near future. The recent demonstration that CRISPR/Cas9 technology can quickly and efficiently generate mouse models in weeks that used to take months (if not years) to create will expedite our ability to study gene functions in laboratory animals [52]. We expect to see the technical feasibility of faster generation of genetically modified animals and more studies on gene interactions by simultaneously knocking out multiple genes. It is clearly an exciting time to be contributing to scientific knowledge via the use of GEMMS. Perhaps even more important is the fact that CRISPR/Cas9 (and related [53]) technology will allow the rapid genetic manipulation of many other model systems that will advance our knowledge of normal development and disease. Provided that this powerful system is handled in an ethical manner [54], it is likely to revolutionize methods to gain biological insights. In fact, it already has done so.

References

1. Joiner DM, et al. LRP5 and LRP6 in development and disease. Trends Endocrinol Metab. 2013;24(1):31–9.
2. Clevers H, Nusse R. Wnt/beta-catenin signaling and disease. Cell. 2012;149(6):1192–205.
3. Gong Y, et al. LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. Cell. 2001;107(4):513–23.
4. Little RD, et al. A mutation in the LDL receptor-related protein 5 gene results in the autosomal dominant high-bone-mass trait. Am J Hum Genet. 2002;70(1):11–9.
5. Boyden LM, et al. High bone density due to a mutation in LDL-receptor-related protein 5. N Engl J Med. 2002;346(20):1513–21.
6. Van Wesenbeeck L, et al. Six novel missense mutations in the LDL receptor-related protein 5 (LRP5) gene in different conditions with an increased bone density. Am J Hum Genet. 2003;72(3):763–71.
7. Mani A, et al. LRP6 mutation in a family with early coronary disease and metabolic risk factors. Science. 2007;315(5816):1278–82.
8. Balemans W, et al. Increased bone density in sclerosteosis is due to the deficiency of a novel secreted protein (SOST). Hum Mol Genet. 2001;10(5):537–43.
9. van Bezooijen RL, et al. Sclerostin is an osteocyte-expressed negative regulator of bone for-

mation, but not a classical BMP antagonist. J Exp Med. 2004;199(6):805–14.
10. Johnson EB, Hammer RE, Herz J. Abnormal development of the apical ectodermal ridge and polysyndactyly in Megf7-deficient mice. Hum Mol Genet. 2005;14(22):3523–38.
11. Xiong L, et al. Lrp4 in osteoblasts suppresses bone formation and promotes osteoclastogenesis and bone resorption. Proc Natl Acad Sci U S A. 2015;112(11):3487–92.
12. Leupin O, et al. Bone overgrowth-associated mutations in the LRP4 gene impair sclerostin facilitator function. J Biol Chem. 2011;286(22):19489–500.
13. Mason JJ, Williams BO. SOST and DKK: antagonists of LRP family signaling as targets for treating bone disease. J Osteoporos. 2010;2010, 460120.
14. Rey JP, Ellies DL. Wnt modulators in the biotech pipeline. Dev Dyn. 2010;239(1):102–14.
15. Padhi D, et al. Single-dose, placebo-controlled, randomized study of AMG 785, a sclerostin monoclonal antibody. J Bone Miner Res. 2011;26(1):19–26.
16. McColm J, et al. Single- and multiple-dose randomized studies of blosozumab, a monoclonal antibody against sclerostin, in healthy postmenopausal women. J Bone Miner Res. 2014;29(4):935–43.
17. Palmiter RD, Brinster RL. Germ-line transformation of mice. Annu Rev Genet. 1986;20:465–99.
18. Hogan B. A shared vision. Dev Cell. 2007;13(6):769–71.
19. Thomas KR, Capecchi MR. Targeted disruption of the murine int-1 proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development. Nature. 1990;346(6287):847–50.
20. Takada S, et al. Wnt-3a regulates somite and tailbud formation in the mouse embryo. Genes Dev. 1994;8(2):174–89.
21. Parr BA, McMahon AP. Dorsalizing signal Wnt-7a required for normal polarity of D-V and A-P axes of mouse limb. Nature. 1995;374(6520):350–3.
22. Hamilton DL, Abremski K. Site-specific recombination by the bacteriophage P1 lox-Cre system. *Cre*-mediated synapsis of two lox sites. J Mol Biol. 1984;178(2):481–6.
23. Nagy A. Cre recombinase: the universal reagent for genome tailoring. Genesis. 2000;26(2):99–109.
24. Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet. 1999;21(1):70–1.
25. Muzumdar MD, et al. A global double-fluorescent Cre reporter mouse. Genesis. 2007;45(9):593–605.
26. Zhang M, et al. Osteoblast-specific knockout of the insulin-like growth factor (IGF) receptor gene reveals an essential role of IGF signaling in bone matrix mineralization. J Biol Chem. 2002;277(46):44005–12.
27. Zhong ZA, et al. Wntless spatially regulates bone development through beta-catenin-dependent and independent mechanisms. Dev Dyn. 2015;244(10):1347–55.
28. Holmen SL, et al. Essential role of beta-catenin in postnatal bone acquisition. J Biol Chem. 2005;280(22):21162–8.
29. Regard JB, et al. Wnt signaling in bone development and disease: making stronger bone with Wnts. Cold Spring Harb Perspect Biol. 2012;4(12).
30. Glass, D.A.2nd, , et al., Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation. Dev Cell, 2005. 8(5): p. 751-764.
31. Yadav VK, et al. Lrp5 controls bone formation by inhibiting serotonin synthesis in the duodenum. Cell. 2008;135(5):825–37.
32. Kode A, et al. Lrp5 regulation of bone mass and serotonin synthesis in the gut. Nat Med. 2014;20(11):1228–9.
33. Cui Y, et al. Reply to Lrp5 regulation of bone mass and gut serotonin synthesis. Nat Med. 2014;20(11):1229–30.
34. Cui Y, et al. Lrp5 functions in bone to regulate bone mass. Nat Med. 2011;17(6):684–91.
35. Riddle RC, et al. Lrp5 and Lrp6 exert overlapping functions in osteoblasts during postnatal bone acquisition. PLoS One. 2013;8(5):e63323.

36. Shen J, Chen D. Recent progress in osteoarthritis research. J Am Acad Orthop Surg. 2014;22(7):467–8.
37. Zhu M, et al. Activation of beta-catenin signaling in articular chondrocytes leads to osteoarthritis-like phenotype in adult beta-catenin conditional activation mice. J Bone Miner Res. 2009;24(1):12–21.
38. Ono N, et al. A subset of chondrogenic cells provides early mesenchymal progenitors in growing bones. Nat Cell Biol. 2014;16(12):1157–67.
39. Day TF, et al. Wnt/beta-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. Dev Cell. 2005;8(5):739–50.
40. Hill TP, et al. Canonical Wnt/beta-catenin signaling prevents osteoblasts from differentiating into chondrocytes. Dev Cell. 2005;8(5):727–38.
41. Zhu M, et al. Inhibition of beta-catenin signaling in articular chondrocytes results in articular cartilage destruction. Arthritis Rheum. 2008;58(7):2053–64.
42. Wei W, et al. Biphasic and dosage-dependent regulation of osteoclastogenesis by beta-catenin. Mol Cell Biol. 2011;31(23):4706–19.
43. Weivoda MM, et al. Wnt Signaling inhibits osteoclast differentiation by activating canonical and noncanonical cAMP/PKA pathways. J Bone Miner Res. 2016;31(1):65–75.
44. Winkler DG, et al. Osteocyte control of bone formation via sclerostin, a novel BMP antagonist. EMBO J. 2003;22(23):6267–76.
45. Brunkow ME, et al. Bone dysplasia sclerosteosis results from loss of the SOST gene product, a novel cystine knot-containing protein. Am J Hum Genet. 2001;68(3):577–89.
46. Maretto S, et al. Mapping Wnt/beta-catenin signaling during mouse development and in colorectal tumors. Proc Natl Acad Sci U S A. 2003;100(6):3299–304.
47. Zhong Z, et al. Wntless functions in mature osteoblasts to regulate bone mass. Proc Natl Acad Sci U S A. 2012;109(33):E2197–204.
48. Bassett JH, et al. Rapid-throughput skeletal phenotyping of 100 knockout mice identifies 9 new genes that determine bone strength. PLoS Genet. 2012;8(8):e1002858.
49. Zhang X, et al. Notum is required for neural and head induction via Wnt deacylation, oxidation, and inactivation. Dev Cell. 2015;32(6):719–30.
50. Kakugawa S, et al. Notum deacylates Wnt proteins to suppress signalling activity. Nature. 2015;519(7542):187–92.
51. Brommage R. Genetic Approaches To Identifying Novel Osteoporosis Drug Targets. J Cell Biochem. 2015;116(10):2139–45.
52. Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering. Cell. 2014;157(6):1262–78.
53. Zetsche B, et al. Cpf1 Is a single RNA-guided endonuclease of a Class 2 CRISPR-Cas system. Cell. 2015;163(3):759–71.
54. Baltimore D, et al. Biotechnology. A prudent path forward for genomic engineering and germline gene modification. Science. 2015;348(6230):36–8.
55. Joeng KS, et al. The swaying mouse as a model of osteogenesis imperfecta caused by WNT1 mutations. Hum Mol Genet. 2014;23(15):4035–42.
56. Laine CM, et al. WNT1 mutations in early-onset osteoporosis and osteogenesis imperfecta. N Engl J Med. 2013;368(19):1809–16.
57. Takada I, et al. A histone lysine methyltransferase activated by non-canonical Wnt signalling suppresses PPAR-gamma transactivation. Nat Cell Biol. 2007;9(11):1273–85.
58. Greco TL, et al. Analysis of the vestigial tail mutation demonstrates that Wnt-3a gene dosage regulates mouse axial development. Genes Dev. 1996;10(3):313–24.
59. Stark K, et al. Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. Nature. 1994;372(6507):679–83.
60. Spater D, et al. Wnt9a signaling is required for joint integrity and regulation of Ihh during chondrogenesis. Development. 2006;133(15):3039–49.
61. Yamaguchi TP, et al. A Wnt5a pathway underlies outgrowth of multiple structures in the vertebrate embryo. Development. 1999;126(6):1211–23.

62. Yang Y, et al. Wnt5a and Wnt5b exhibit distinct activities in coordinating chondrocyte proliferation and differentiation. Development. 2003;130(5):1003–15.
63. Parr BA, et al. The classical mouse mutant postaxial hemimelia results from a mutation in the Wnt 7a gene. Dev Biol. 1998;202(2):228–34.
64. Juriloff DM, et al. Wnt9b is the mutated gene involved in multifactorial nonsyndromic cleft lip with or without cleft palate in A/WySn mice, as confirmed by a genetic complementation test. Birth Defects Res A Clin Mol Teratol. 2006;76(8):574–9.
65. Bennett CN, et al. Regulation of osteoblastogenesis and bone mass by Wnt10b. Proc Natl Acad Sci U S A. 2005;102(9):3324–9.
66. Stevens JR, et al. Wnt10b deficiency results in age-dependent loss of bone mass and progressive reduction of mesenchymal progenitor cells. J Bone Miner Res. 2010;25(10):2138–47.
67. Zheng HF, et al. WNT16 influences bone mineral density, cortical bone thickness, bone strength, and osteoporotic fracture risk. PLoS Genet. 2012;8(7):e1002745.
68. Moverare-Skrtic S, et al. Osteoblast-derived WNT16 represses osteoclastogenesis and prevents cortical bone fragility fractures. Nat Med. 2014;20(11):1279–88.
69. Yu H, et al. Frizzled 1 and frizzled 2 genes function in palate, ventricular septum and neural tube closure: general implications for tissue fusion processes. Development. 2010;137(21):3707–17.
70. Albers J, et al. Canonical Wnt signaling inhibits osteoclastogenesis independent of osteoprotegerin. J Cell Biol. 2013;200(4):537–49.
71. Albers J, et al. Control of bone formation by the serpentine receptor Frizzled-9. J Cell Biol. 2011;192(6):1057–72.
72. Iwaniec UT, et al. PTH stimulates bone formation in mice deficient in Lrp5. J Bone Miner Res. 2007;22(3):394–402.
73. Clement-Lacroix P, et al. Lrp5-independent activation of Wnt signaling by lithium chloride increases bone formation and bone mass in mice. Proc Natl Acad Sci U S A. 2005;102(48):17406–11.
74. Kato M, et al. Cbfa1-independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in Lrp5, a Wnt coreceptor. J Cell Biol. 2002;157(2):303–14.
75. Holmen SL, et al. Decreased BMD and limb deformities in mice carrying mutations in both Lrp5 and Lrp6. J Bone Miner Res. 2004;19(12):2033–40.
76. Pinson KI, et al. An LDL-receptor-related protein mediates Wnt signalling in mice. Nature. 2000;407(6803):535–8.
77. Carter M, et al. Crooked tail (Cd) model of human folate-responsive neural tube defects is mutated in Wnt coreceptor lipoprotein receptor-related protein 6. Proc Natl Acad Sci U S A. 2005;102(36):12843–8.
78. Kokubu C, et al. Skeletal defects in ringelschwanz mutant mice reveal that Lrp6 is required for proper somitogenesis and osteogenesis. Development. 2004;131(21):5469–80.
79. Kubota T, et al. Lrp6 hypomorphic mutation affects bone mass through bone resorption in mice and impairs interaction with Mesd. J Bone Miner Res. 2008;23(10):1661–71.
80. Karner CM, et al. Lrp4 regulates initiation of ureteric budding and is crucial for kidney formation--a mouse model for Cenani-Lenz syndrome. PLoS One. 2010;5(4):e10418.
81. Simon-Chazottes D, et al. Mutations in the gene encoding the low-density lipoprotein receptor LRP4 cause abnormal limb development in the mouse. Genomics. 2006;87(5):673–7.
82. Weatherbee SD, Anderson KV, Niswander LA. LDL-receptor-related protein 4 is crucial for formation of the neuromuscular junction. Development. 2006;133(24):4993–5000.
83. Choi HY, et al. Lrp4, a novel receptor for Dickkopf 1 and sclerostin, is expressed by osteoblasts and regulates bone growth and turnover in vivo. PLoS One. 2009;4(11):e7930.
84. Fu J, et al. Reciprocal regulation of Wnt and Gpr177/mouse Wntless is required for embryonic axis formation. Proc Natl Acad Sci U S A. 2009;106(44):18598–603.
85. Morvan F, et al. Deletion of a single allele of the Dkk1 gene leads to an increase in bone formation and bone mass. J Bone Miner Res. 2006;21(6):934–45.

86. Mukhopadhyay M, et al. Dickkopf1 is required for embryonic head induction and limb morphogenesis in the mouse. Dev Cell. 2001;1(3):423–34.
87. Li X, et al. Dkk2 has a role in terminal osteoblast differentiation and mineralized matrix formation. Nat Genet. 2005;37(9):945–52.
88. Li C, et al. Increased callus mass and enhanced strength during fracture healing in mice lacking the sclerostin gene. Bone. 2011;49(6):1178–85.
89. Li X, et al. Targeted deletion of the sclerostin gene in mice results in increased bone formation and bone strength. J Bone Miner Res. 2008;23(6):860–9.
90. Niziolek PJ, et al. High-bone-mass-producing mutations in the Wnt signaling pathway result in distinct skeletal phenotypes. Bone. 2011;49(5):1010–9.
91. Bodine PV, et al. The Wnt antagonist secreted frizzled-related protein-1 is a negative regulator of trabecular bone formation in adult mice. Mol Endocrinol. 2004;18(5):1222–37.
92. Morello R, et al. Brachy-syndactyly caused by loss of Sfrp2 function. J Cell Physiol. 2008;217(1):127–37.
93. Perry WL 3rd, et al. Phenotypic and molecular analysis of a transgenic insertional allele of the mouse Fused locus. Genetics. 1995;141(1):321–32.
94. Vasicek TJ, et al. Two dominant mutations in the mouse fused gene are the result of transposon insertions. Genetics. 1997;147(2):777–86.
95. Dao DY, et al. Axin2 regulates chondrocyte maturation and axial skeletal development. J Orthop Res. 2010;28(1):89–95.
96. Yan Y, et al. Axin2 controls bone remodeling through the beta-catenin-BMP signaling pathway in adult mice. J Cell Sci. 2009;122(Pt 19):3566–78.
97. Yu HM, et al. The role of Axin2 in calvarial morphogenesis and craniosynostosis. Development. 2005;132(8):1995–2005.
98. Qian L, et al. Tissue-specific roles of Axin2 in the inhibition and activation of Wnt signaling in the mouse embryo. Proc Natl Acad Sci U S A. 2011;108(21):8692–7.
99. Itoh S, et al. GSK-3alpha and GSK-3beta proteins are involved in early stages of chondrocyte differentiation with functional redundancy through RelA protein phosphorylation. J Biol Chem. 2012;287(35):29227–36.
100. Hoeflich KP, et al. Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. Nature. 2000;406(6791):86–90.
101. Kugimiya F, et al. GSK-3beta controls osteogenesis through regulating Runx2 activity. PLoS One. 2007;2(9):e837.
102. Nelson ER, et al. Role of GSK-3beta in the osteogenic differentiation of palatal mesenchyme. PLoS One. 2011;6(10):e25847.
103. Joeng KS, et al. Lrp5 and Lrp6 redundantly control skeletal development in the mouse embryo. Dev Biol. 2011;359(2):222–9.
104. Guo X, et al. Wnt/beta-catenin signaling is sufficient and necessary for synovial joint formation. Genes Dev. 2004;18(19):2404–17.
105. Soshnikova N, et al. Genetic interaction between Wnt/beta-catenin and BMP receptor signaling during formation of the AER and the dorsal-ventral axis in the limb. Genes Dev. 2003;17(16):1963–8.
106. Hu H, et al. Sequential roles of Hedgehog and Wnt signaling in osteoblast development. Development. 2005;132(1):49–60.
107. Rodda SJ, McMahon AP. Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors. Development. 2006;133(16):3231–44.
108. Kramer I, et al. Osteocyte Wnt/beta-catenin signaling is required for normal bone homeostasis. Mol Cell Biol. 2010;30(12):3071–85.
109. Dao DY, et al. Cartilage-specific beta-catenin signaling regulates chondrocyte maturation, generation of ossification centers, and perichondrial bone formation during skeletal development. J Bone Miner Res. 2012;27(8):1680–94.
110. Chen J, Long F. β-catenin promotes bone formation and suppresses bone resorption in post-

natal growing mice. J Bone Miner Res. 2013;8(5):1160–9.
111. Barrott JJ, et al. Deletion of mouse Porcn blocks Wnt ligand secretion and reveals an ectodermal etiology of human focal dermal hypoplasia/Goltz syndrome. Proc Natl Acad Sci U S A. 2011;108(31):12752–7.
112. Liu W, et al. Deletion of Porcn in mice leads to multiple developmental defects and models human focal dermal hypoplasia (Goltz syndrome). PLoS One. 2012;7(3):e32331.
113. Zhu X, et al. Wls-mediated Wnts differentially regulate distal limb patterning and tissue morphogenesis. Dev Biol. 2012;365(2):328–38.
114. Maruyama T, Jiang M, Hsu W. Gpr177, a novel locus for bone-mineral-density and osteoporosis, regulates osteogenesis and chondrogenesis in skeletal development. J Bone Miner Res. 2013;28(5):1150–9.
115. Lu C, et al. Wnt-mediated reciprocal regulation between cartilage and bone development during endochondral ossification. Bone. 2013;53(2):566–74.
116. Akiyama H, et al. Interactions between Sox9 and beta-catenin control chondrocyte differentiation. Genes Dev. 2004;18(9):1072–87.
117. Mirando AJ, et al. beta-catenin/cyclin D1 mediated development of suture mesenchyme in calvarial morphogenesis. BMC Dev Biol. 2010;10:116.
118. Lee HH, Behringer RR. Conditional expression of Wnt4 during chondrogenesis leads to dwarfism in mice. PLoS One. 2007;2(5):e450.
119. Popperl H, et al. Misexpression of Cwnt8C in the mouse induces an ectopic embryonic axis and causes a truncation of the anterior neuroectoderm. Development. 1997;124(15):2997–3005.
120. Oh H, Chun CH, Chun JS. Dkk-1 expression in chondrocytes inhibits experimental osteoarthritic cartilage destruction in mice. Arthritis Rheum. 2012;64(8):2568–78.
121. Oh H, et al. Misexpression of Dickkopf-1 in endothelial cells, but not in chondrocytes or hypertrophic chondrocytes, causes defects in endochondral ossification. J Bone Miner Res. 2012;27(6):1335–44.
122. Yao GQ, et al. Targeted overexpression of Dkk1 in osteoblasts reduces bone mass but does not impair the anabolic response to intermittent PTH treatment in mice. J Bone Miner Metab. 2011;29(2):141–8.
123. Cho HY, et al. Transgenic mice overexpressing secreted frizzled-related proteins (sFRP)4 under the control of serum amyloid P promoter exhibit low bone mass but did not result in disturbed phosphate homeostasis. Bone. 2010;47(2):263–71.
124. Nakanishi R, et al. Osteoblast-targeted expression of Sfrp4 in mice results in low bone mass. J Bone Miner Res. 2008;23(2):271–7.
125. Mikasa M, et al. Regulation of Tcf7 by Runx2 in chondrocyte maturation and proliferation. J Bone Miner Metab. 2011;29(3):291–9.
126. Hoeppner LH, et al. Lef1DeltaN binds beta-catenin and increases osteoblast activity and trabecular bone mass. J Biol Chem. 2011;286(13):10950–9.

Chapter 2
Role of the Receptor-Mediated Signaling Pathways on the Proliferation and Differentiation of Pluripotent Stem Cells

Toshiaki Ishizuka

Abstract Several receptor-mediated signaling pathways are involved in the self-renewal or differentiation of pluripotent stem cells such as embryonic stem (ES) cells or induced pluripotent stem (iPS) cells. The activation of the JAK/STAT pathway induced by leukemia inhibitory factor (LIF) plays a critical role in the self-renewal of mouse ES or iPS cells. However, it has been demonstrated that fibroblast growth factor 2 (FGF2) maintains self-renewal of human ES or iPS cells by supporting stable expression of the extracellular matrix proteins through the activation of the PI3K/Akt pathway. Recent studies confirm that both the MEK/ERK and the PI3K/Akt pathways are involved in the self-renewal of both mouse ES cells and iPS cells. We have also revealed that stimulation of either α_1-adrenoceptor or angiotensin II type 1 receptor (AT_1R) leads to an increase in human iPS cell proliferation via Gq-dependent MEK/ERK and PI3K/Akt signaling pathways independent of FGF2.

Activation of the Smad signaling pathway by bone morphogenetic proteins (BMPs) and activin/nodal has been shown to promote cardiovascular differentiation of mouse and human ES or iPS cells. In addition, treatment with isoproterenol (a β-adrenoceptor agonist) enhances the cardiovascular differentiation of human iPS cells exposed to activin A, BMP4, and FGF2. As stimulation with β-adrenoceptors promotes cAMP and PKA activation, the cardiovascular differentiation of the cells may be enhanced by cAMP/PKA-dependent signaling pathways.

It has been found that treatment with retinoic acid (RA) during embryoid body (EB) formation induces the differentiation of mouse ES cells into neural progenitor cells (NPCs). RA treatment increases the level of active cAMP response element-binding (CREB) protein by enhancing the activity of c-Jun N-terminal kinase (JNK). It has been revealed that stimulation of either β-adrenoceptors or $5\text{-}HT_4$ receptors enhances the RA-induced differentiation of mouse iPS cells into NPCs through activation of the cAMP/PKA signaling pathway and the enhancement of CREB phosphorylation.

T. Ishizuka
Department of Pharmacology, National Defense Medical College, Saitama, Japan
e-mail: tishizu@ndmc.ac.jp

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N. Shinomiya et al. (eds.), *Regulation of Signal Transduction in Human Cell Research*, Current Human Cell Research and Applications,
https://doi.org/10.1007/978-981-10-7296-3_2

This review focuses on the role of the receptor-mediated signaling pathways in the proliferation and differentiation of pluripotent stem cells. Understanding the receptor-mediated signaling pathways that influence the proliferation and differentiation of pluripotent stem cells may be useful in the development of culture conditions that promote the therapeutic effects of regenerative medicine.

Keywords Guanine nucleotide-binding protein-coupled receptors (GPCR) • Pluripotent stem cells • Extracellular signal-regulated kinase (ERK) • PI3K/Akt • cAMP/PKA

2.1 Introduction

As embryonic stem (ES) cells derived from the inner cell mass of mammalian blastocysts have the ability to grow indefinitely while maintaining pluripotency [1, 2], human ES cells are expected to prove useful in understanding disease mechanisms, screening for safe and effective drugs, and treating patients with various diseases and injuries [3]. However, the use of human embryos to generate ES cells faces ethical concerns and risk of immunogenicity/allograft rejection. To circumvent the therapeutic limitations of human ES cells, Takahashi et al. [4] generated induced pluripotent stem (iPS) cells from adult human dermal fibroblasts by the retrovirus-mediated transfection of four transcription factors, namely, Oct3/4, Sox2, c-Myc, and Klf4. They also demonstrated that human iPS cells are similar to human ES cells in morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes, and differentiation into cell types of the three germ layers in vitro. However, the reprogramming efficiency of human somatic cells into iPS cells is very low in spite of the development of modified methods. In addition, the capacity for human iPS cells to differentiate into poorly regenerated cells, such as neural cells or cardiomyocytes, is also low. Thus, understanding of the mechanisms controlling the signaling pathways in the processes of reprogramming or differentiation may offer therapeutic advantages [5].

2.2 Role of the Receptor Signaling Pathways on Proliferation of Pluripotent Stem Cells

Smith and Hooper [6] and Smith et al. [7] demonstrated that self-renewal of mouse ES cells is dependent on paracrine signals from feeder cells such as mouse embryonic fibroblasts (MEF) on which the cells were cultured. It has also been established that the factor required for self-renewal of mouse ES cells is leukemia inhibitory factor (LIF) [8, 9]. LIF is a member of the family of interleukin-6 (IL-6)-type

cytokines and binds directly to the LIF receptor-β (LIFRβ) that subsequently heterodimerizes with the signal transducer glycoprotein 130 (gp130) [10]. The formation of a trimeric complex composed of LIFRβ, gp130, and LIF leads to the activation of Janus kinase – the signal transducer and activator of the transcription (JAK/STAT) and mitogen-activated protein kinase (MAPK) pathways, which are essential for the proliferation of mouse ES cells [11]. However, several reports have suggested that the proliferation of mouse ES cells is mediated by the signaling pathways activated by other receptor types such as receptor tyrosine kinases (RTK), guanine nucleotide-binding protein-coupled receptors (GPCR), and wingless-typemouse mammary virus tumor integration site family (Wnt) receptors.

Kim et al. [12] revealed that the epinephrine-enhanced proliferation of mouse ES cells cultured with LIF is mainly through both α- and β-adrenoceptors, which are the most common GPCR. They also showed that α_{1A}-, α_{2A}-, β_1-, β_2-, and β_3-adrenoceptors were expressed in the cells. It is well known that stimulation of α_1-adrenoceptors couples the receptor to guanine nucleotide-binding protein q polypeptide (Gq) protein. This coupling to Gq activates phospholipase C (PLC), leading to an increase in inositol-1,4,5-triphosphate (IP_3) and 1,2-diacyl glycerol (DAG), both of which induce intracellular calcium elevation and protein kinase C (PKC) activation [13]. Conversely, β-adrenoceptor stimulation activates the stimulatory alpha subunit of guanine nucleotide-binding protein (Gs) and adenylyl cyclase, which promote cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) activation [14]. Kim et al. [12] suggested that the activation of extracellular signal-regulated kinase (ERK) is mainly involved in the epinephrine-induced proliferation of cells via either α_1-adrenoceptor-dependent PKC or β-adrenoceptor-dependent PKA. In addition, Prenzel et al. [15] showed that activation of either α_1- or β-adrenoceptors leads to the extracellular release of epidermal growth factor (EGF) receptor ligands at the cell surface by matrix metalloproteases. Kim et al. [12] also observed that epinephrine activates the PI3K/Akt pathway via α_1- or β-adrenoceptor transactivation of the EGF receptor in mouse ES cells. These findings suggest that epinephrine stimulates proliferation via ERK through cAMP/PKA, Ca^{2+}/PKC, and PI3K/Akt signaling pathways in mouse ES cells.

Han et al. [16] showed that angiotensin II (Ang II)-induced increase of DNA synthesis and proliferation in mouse ES cells cultured with LIF were due to the stimulation of angiotensin type 1 receptors (AT_1R). The activation of AT_1Rs, which are Gq-coupled receptors, induced inositol phosphate formation and Ca^{2+}/PKC-dependent ERK activation. They also revealed that Ang II activates the PI3K/Akt pathway via AT_1R transactivation of EGF receptor in mouse ES cells. Thus, their results indicate that Ang II-induced DNA synthesis and proliferation may require two parallel signaling pathways: (1) AT_1R-mediated Ca^{2+}/PKC/ERK and (2) AT_1R-induced transactivation of EGF receptor-dependent PI3K/Akt.

Ishizuka and Watanabe [17] reported that stimulation with a selective α_1-adrenoceptor agonist phenylephrine increases DNA synthesis and proliferation of mouse iPS cells cultured in a medium with LIF. The study showed that the phosphorylation of either ERK or Akt in the cells cultured with LIF was

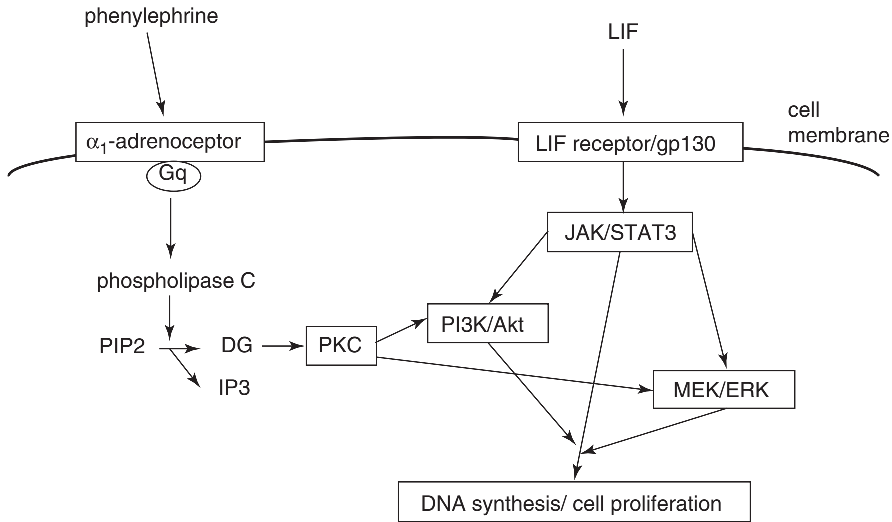

Fig. 2.1 Mechanism of the signaling pathways involved in the DNA synthesis and proliferation of mouse pluripotent stem cells. Gq, guanosine 5′-triphosphate-binding protein q; PIP2, phosphatidylinositol-4,5-bisphosphate; DG, diacylglycerol; IP3, inositol-1,4,5-triphosphate

enhanced by phenylephrine, and a selective α_1-adrenoceptor antagonist blocked the enhancement. DNA synthesis enhanced by phenylephrine is inhibited by either a PI3K inhibitor or a MEK inhibitor. Conversely, under LIF-free conditions, phenylephrine treatment slightly increased phosphorylation of ERK or Akt and did not affect DNA synthesis. These findings suggest that phenylephrine-induced phosphorylation of ERK or Akt, without LIF-induced JAK/STAT activation, may be insufficient for DNA synthesis in mouse iPS cells. However, stimulation with α_1-adrenoceptors in the presence of LIF may augment phosphorylation of ERK or Akt synergically with LIF-induced JAK/STAT activation and enhance DNA synthesis (Fig. 2.1). Thus, these results confirm that both MEK/ERK and PI3K/Akt are self-renewal-mediating pathways in both mouse ES cells and iPS cells.

Landgraf et al. [18] showed that mouse ES cells express nicotinic acetylcholine receptors (nAchR). Several previous studies suggest that stimulation with nAchR enhances the growth of endothelial progenitor cells [19, 20]. We revealed by immunofluorescence staining that α_4-nAchR and α_7-nAchR are expressed on mouse iPS cells [21]. Treatment with nicotine increases DNA synthesis of the cells, which is inhibited by pretreatment with antagonists of either α_4-nAchR or α_7-nAchR. In addition, treatment with nicotine increases the intracellular Ca^{2+} level and calmodulin-dependent protein kinase II (CaMKII) phosphorylation. However, treatment with nicotine does not affect the phosphorylation of either ERK, Akt, or JAK/

STAT in the cells. Thus, the CaMKII signaling pathway may be involved in the proliferation of mouse iPS cells without affecting MEK/ERK, PI3K/Akt, or JAK/STAT.

Daheron et al. [22] reported that LIF cannot sustain self-renewal of human ES cells. In addition, it has been shown that the JAK/STAT pathway is not essential for maintaining pluripotency and self-renewal in human ES cells [23, 24]. However, Kim et al. [25] have demonstrated that fibroblast growth factor 2 (FGF2) maintains the self-renewal of human ES cells by supporting stable expression of the extracellular matrix proteins through the activation of the PI3K/Akt pathway. We revealed that treatment with either phenylephrine (a selective α_1-adrenoceptor agonist) or Ang II enhances DNA synthesis in human iPS cells cultured in the absence of FGF2 [26]. We also showed that the enhancement of DNA synthesis by either phenylephrine or Ang II is suppressed by pretreatment with PD98059 (a MEK inhibitor) and LY294002 (a PI3K inhibitor). Moreover, the phosphorylation of Akt and ERK in the cells is enhanced by either phenylephrine or Ang II. Both α_1- adrenoceptor and AT_1R are Gq-coupled receptors. In this study, siRNA directed against Gq inhibited DNA synthesis and the phosphorylation of ERK and Akt induced by phenylephrine or Ang II. Thus, activation of α_1-adrenoceptor or AT_1R may lead to an increase in the proliferation of human iPS cells via Gq-dependent MEK/ERK and PI3K/Akt signaling pathways independent of FGF2 (Fig. 2.2).

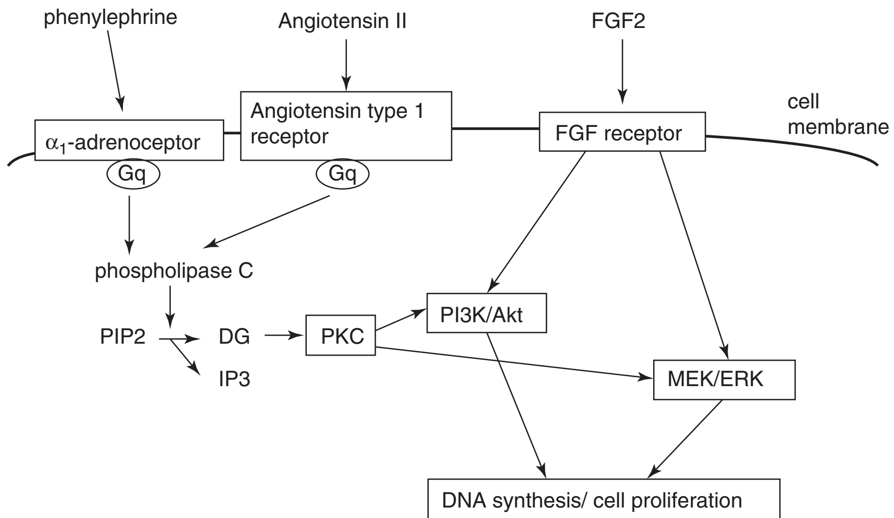

Fig. 2.2 Mechanism of the signaling pathways involved in the DNA synthesis and proliferation of human pluripotent stem cells

2.3 Role of Receptor Signaling Pathways in Differentiation of Pluripotent Stem Cells into Cardiovascular Progenitor Cells

Bone morphogenetic proteins (BMPs) belong to a transforming growth factor-β (TGFβ) superfamily and play critical roles in cell differentiation during embryonic development [27]. BMPs activate Smad 1/5/8 signaling via activin receptor-like kinase (ALK)1, ALK2, ALK3, and ALK6. TGFβ, activin, and nodal activate Smad 2/3 via ALK4, ALK5, and ALK7 [27, 28]. Although TGFβ, activin, and nodal maintain pluripotency of human ES cells [29, 30], blockade of BMP signaling is essential to maintain pluripotency [31, 32]. It has been revealed that the activation of the BMP signaling pathway promotes differentiation of human ES cells into mesoderm [33] or hematopoietic cells [34]. Several studies have demonstrated that stimulation with BMP and TGFβ/activin/nodal promotes cardiovascular differentiation during embryonic development [35, 36] and cardiovascular differentiation from ES cells [37]. In the mouse embryo and the mouse ES cell differentiation model, Flk-1^{+} (fetal liver kinase-1, also known as kinase insert domain protein receptor (KDR)) cells are identified as cardiovascular progenitor cells which display the capacity to generate cardiomyocytes, vascular endothelial cells, and vascular smooth muscle cells [38]. Kattman et al. [39] also reported that combined stimulation of activin A and BMP4 induces Flk-1^{+} cells in serum-free cultures of embryoid bodies (EB) generated from mouse ES cells or iPS cells. Yang et al. [40] showed that after induction with combinations of activin A, BMP4, FGF2, VEGF, and dickkopf homolog 1 (DKK1, an inhibitor of Wnt signaling), human ES cell-derived EBs generate KDR^{+} cardiovascular progenitor cells. On the other hand, it has been documented that KDR^{+} cells can be induced from human ES cells or iPS cells after stimulation with activin A and BMP4 [39].

Bai et al. [41] found that combined stimulations of BMP2, BMP4, BMP7, FGF2, and VEGF promote differentiation of human ES cells into CD34^{+}CD31^{+} cells, which have the potential to give rise to vascular endothelial cells and smooth muscle cells. As the pretreatment with dorsomorphin, an inhibitor of Smad 1/5/8 phosphorylation, blocked the differentiation into CD34^{+}CD31^{+} cells, BMP/ Smad signaling may be critical for vascular progenitor development of human ES cells (Fig. 2.3).

Yan et al. [42] reported that stimulation of mouse ES cell-derived EB with isoproterenol (a nonspecific β-adrenoceptor agonist) enhanced their differentiation into cardiomyocytes. In addition, they found that the effect of isoproterenol was eliminated by pretreatment with a β_1-, β_2-adrenoceptor antagonist, or SB203580 (a specific p38 MAPK inhibitor). It has been revealed that the culture of mouse EB with dibutyryl-cAMP (a cAMP analogue) enhances cardiac differentiation [43] or retinoic acid-induced differentiation into vascular smooth muscle cells [44]. We have demonstrated the involvement of β-adrenoceptors in the differentiation of human iPS cells into cardiovascular progenitor cells [45]. The induction of differentiation into KDR^{+} cells was performed on feeder cells in a differentiation medium

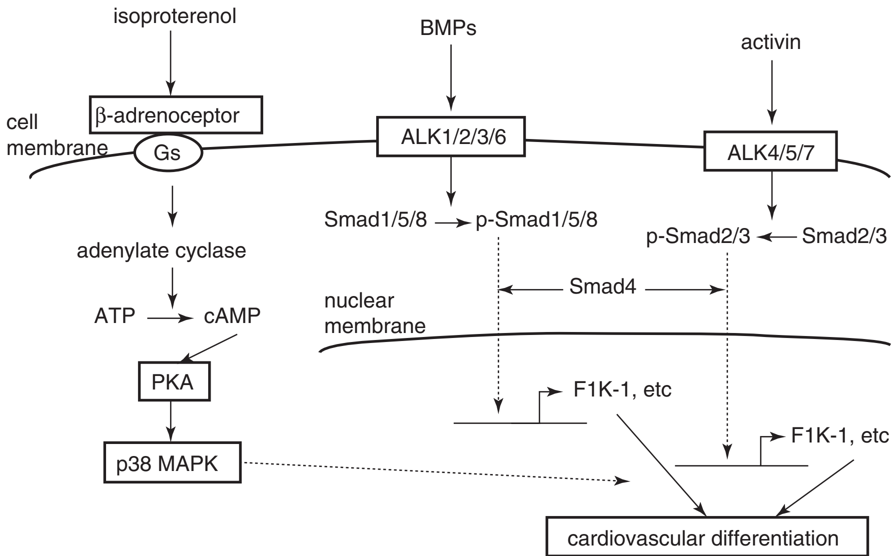

Fig. 2.3 Mechanism of the signaling pathways involved in the cardiovascular differentiation of mouse/human pluripotent stem cells. Gq, guanosine 5′-triphosphate binding protein s; ATP, adenosine 5′-triphosphate; cAMP, cyclic adenosine 3′,5′-monophosphate; ALK, activin-like kinase

with activin A, BMP4, and FGF2. When the iPS cells that were exposed to activin A, BMP4, and FGF2 were treated with isoproterenol, the expression of KDR was increased compared to that of the cells without isoproterenol. Pretreatment with either a β1- or β2-adrenoceptor antagonist inhibited the isoproterenol-induced increase in KDR expression. In addition, pretreatment with both H89 (a PKA inhibitor) and SB203580 (a specific p38 MAPK inhibitor) inhibited the effect of isoproterenol. Stimulation of β-adrenoceptors, which are Gs-coupled receptors, promotes cAMP and PKA activation [14]. These findings suggest that cardiovascular differentiation of both ES cells and iPS cells is enhanced by cAMP-/PKA-dependent activation of p38 MAPK (Fig. 2.3).

2.4 Role of Receptor Signaling Pathways in the Differentiation of Pluripotent Stem Cells into Neural Progenitor Cells

Retinoic acid (RA) is the biologically active form of vitamin A and has been shown to play an important role during embryogenesis [46]. In particular, RA influences neural development in the early stages of central nervous system development. Shan et al. [47] found that during EB formation, RA induces differentiation of mouse ES cells into neural progenitor cells (NPCs). Thus, RA is thought to be one of the most

important inducers of neural differentiation in mouse ES cells [48]. As RA treatment increases the level of active cAMP response element-binding (CREB) protein by enhancing the activity of c-Jun N-terminal kinase (JNK), Shan et al. [47] suggest that CREB plays a role in RA-induced NPC differentiation. The activity of CREB is triggered by phosphorylation on Ser133, which recruits the CREB-binding protein to the initiator complex and thereby promotes transcription [49]. The phosphorylation of CREB on Ser133 is reported to be induced by cAMP/PKA-, Ca^{2+}-, or the neurotrophic factor-dependent signaling pathway [50].

Di-Gregorio et al. [51] demonstrated that the activation of the BMP signaling pathway inhibits premature neural differentiation of the mouse embryo [51]. In mouse ES cells, BMP signaling has negative effects on neural differentiation [52, 53]. It has been reported that the induction of important downstream proteins of the BMP signaling pathway can inhibit the neural differentiation of ES cells and can sustain self-renewal in collaboration with LIF [54]. Zhang et al. [55] demonstrated that BMP4 inhibits the derivation of epiblast stem cells from mouse ES cells and suppresses the neural commitment of epiblast stem cells and promotes their non-neural differentiation. Okada et al. [48] reported that treatment with noggin (an inhibitor of BMP signals) enhances RA-induced differentiation of mouse ES cells into $nestin^+$ $Sox1^+$ NPCs. In addition, inhibition of activin has been shown to promote neural differentiation of human ES cells and iPS cells [56, 57]. As it is known that BMPs activate Smad 1/5/8 signaling and activin/nodal activates Smad 2/3 [27, 28], it is speculated that Smad signaling may modulate the neural lineage commitment of pluripotent stem cells. However, Zhang et al. [55] showed that BMP4 reduced ERK phosphorylation in mouse ES cells and that FGF2 rescued BMP4-reduced ERK phosphorylation. They also found that FGF2 recovered BMP4-inhibited neural commitment of mouse ES cells. Matulka et al. [58] discovered that the inhibition of the activin/ALK4 pathway by SB431542 not only inhibits the phosphorylation of Smad 2/3 but also enhances the phosphorylation of ERK. They further demonstrated that the activation of ERK signaling occurs in SB431542-induced neural differentiation of mouse ES cells. Thus, suppression of the ERK signaling pathway, by either BMP4 or activin, may partially lead to the inhibition of neural differentiation.

Activation of the cAMP/PKA signaling pathway can facilitate neuronal development by inducing mesenchymal stem cells (MSCs) to differentiate into neural lineage cells [59–61]. Activation of β-adrenoceptors is known to stimulate the Gs and adenylyl cyclase, which promote the cAMP/PKA signaling pathway [14]; we therefore examined the effect of β-adrenoceptor activation on the neural differentiation of mouse iPS cell-derived EBs. Although treatment with isoproterenol (a nonspecific β-adrenoceptor agonist) alone did not affect the expression of nestin (a specific marker for NPCs), isoproterenol enhanced RA-induced nestin expression [62]. Pretreatment of EBs with either atenolol (a specific β_1-adrenoceptor antagonist) or H89 (a PKA inhibitor) inhibited the isoproterenol enhancement of ATRA-induced nestin expression.

It has been suggested that serotonin (5-hydroxytryptamine; 5-HT) stimulates adult hippocampal neurogenesis [63], thereby creating a mechanistic link between the suppression of adult neurogenesis and the pathogenesis of major depression, as well as explaining the mechanism underlying the action of antidepressants [64, 65]. It has also been shown that the activation of 5-HT$_{1A}$ or 5-HT$_2$ receptors promotes proliferation and differentiation of NPCs [66, 67]. We have shown that mouse iPS cells express both 5-HT$_{2A}$ and 5-HT$_4$ receptors and, to a lesser extent, 5-HT$_{1A}$ receptors [68]. Treatment with 5-HT enhances the RA-induced expression of nestin and phosphorylation of CREB. Pretreatment with either GR113808 (a specific 5-HT$_4$ receptor antagonist) or H89 inhibits these effects of 5-HT. As with β-adrenoceptors, 5-HT$_4$ receptors also couple to Gs, which stimulates adenylyl cyclase and promotes the cAMP/PKA signaling pathway. It has been suggested that CREB plays a role in RA-induced differentiation of mouse ES cells into NPCs [47]. Phosphorylation of CREB can be triggered through the activation of the cAMP/PKA signaling pathway [14, 69]. Thus, these findings suggest that the stimulation of either β-adrenoceptors or 5-HT$_4$ receptors enhances RA-induced differentiation of pluripotent stem cells into NPCs through the activation of the cAMP/PKA signaling pathway and the enhancement of CREB phosphorylation (Fig. 2.4).

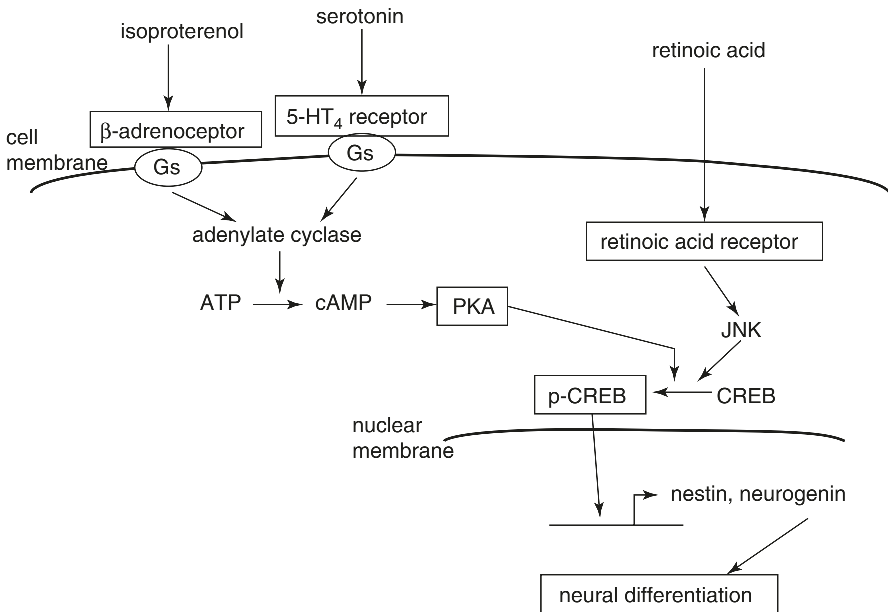

Fig. 2.4 Mechanism of the signaling pathways involved in the neural differentiation of mouse pluripotent stem cells

2.5 Closing Remarks

This review is focused on the role of the receptor-dependent signaling pathways in the proliferation and differentiation of pluripotent stem cells. Establishing a more robust and efficient methodology to propagate tissue stem cells from pluripotent stem cells is critical for these cells to be used as a source of regenerative therapy for tissue damage or tissue injury. Thus, understanding the receptor-dependent signaling pathways which influence the proliferation and differentiation of pluripotent stem cells may be useful in the development of culture conditions that promote the therapeutic effects of regenerative medicine.

Acknowledgments The results of our studies mentioned in this review were supported in part by a Grant-in-Aid for the Special Research Program from the National Defense Medical College and the Scientific Research Program from the Japan Society for the Promotion of Sciences to T.I.

References

1. Evans MJ, Kaufman MH. Establishment in culture of pluripotent stem cells from mouse embryos. Nature. 1981;292:154–6.
2. Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci USA. 1981;78:7634–8.
3. Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. Science. 1998;282:1145–7.
4. Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007;131:861–72.
5. Kane NM, Xiao Q, Baker AH, et al. Pluripotent stem cell differentiation into vascular cells: A novel technology with promises for vascular re(generation). Pharmacol and Therapeutics. 2011;129:29–49.
6. Smith AG, Hooper ML. Buffalo rat liver cells produce a diffusible activity which inhibits the differentiation of murine embryonal carcinoma and embryonic stem cells. Dev Biol. 1987;121:1–9.
7. Smith AG, Heath JK, Donaldson DD, et al. Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. Nature. 1988;336:688–90.
8. Gearing DP, Gough NM, King JA, et al. Molecular cloning and expression of cDNA encoding a murine myeloid leukaemia inhibitory factor (LIF). EMBO J. 1987;6:3995–4002.
9. Williams RL, Hilton DJ, Pease S, et al. Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature. 1988;336:684–7.
10. Kristensen DM, Kalisz M, Nielsen JH. Cytokine signaling in embryonic stem cells. APMIS. 2005;113:756–72.
11. Zhang JG, Owezarek CM, Ward LD, et al. Evidence for the formation of a heterotrimeric complex of leukaemia inhibitory factor with its receptor subunits in solution. Biochem J. 1997;325:693–700.
12. Kim MO, Na SI, Lee MY, et al. Epinephrine increases DNA synthesis via ERK1/2s through cAMP, Ca^{2+}/PKC, and PI3K/Akt signaling pathways in mouse embryonic stem cells. J Cell Biochem. 2008;104:1407–20.
13. Zhong H, Minneman KP. Alpha1-adrenoceptor subtypes. Eur J Pharmacol. 1999;375:261–76.
14. Gonzalez GA, Montminy MR. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine133. Cell. 1989;59:675–80.

15. Prenzel N, Zwick E, Daub H, et al. EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. Nature. 1999;402:884–8.
16. Han HJ, Han JY, Hao JS, et al. Ang II-stimulated DNA synthesis is mediated by Ang II receptor-dependent Ca^{2+}/PKC as well as EGF receptor-dependent PI3K/Akt/mTOR/p70S6K1 signal pathways in mouse embryonic stem cells. J Cell Physiol. 2007;211:618–29.
17. Ishizuka T, Watanabe Y. α_1-adrenoceptor stimulation enhances leukemia inhibitory factor-induced proliferation of mouse-induced pluripotent stem cells. Eur J Pharmacol. 2011;668:42–56.
18. Landgraf D, Barth M, Layer PG, et al. Acetylcholine as a possible signaling molecule in embryonic stem cells: studies on survival, proliferation and death. Chem Biol Interact. 2010;187:115–9.
19. Heeschen C, Jang JJ, Weis M, et al. Nicotine stimulates angiogenesis and promotes tumor growth and atherosclerosis. Nat Med. 2001;7:833–9.
20. Heeschen C, Chang E, Aicher A, et al. Endothelial progenitor cells participate in nicotine-mediated angiogenesis. J Am Coll Cardiol. 2006;48:2553–60.
21. Ishizuka T, Ozawa A, Goshima H, et al. Involvement of nicotinic acetylcholine receptors in the proliferation of mouse induced pluripotent stem cells. Life Sciences. 2012;90:637–48.
22. Daheron L, Opitz SL, Zaehres H, et al. LIF/STAT3 signaling fails to maintain self-renewal of human embryonic stem cells. Stem Cells. 2004;22:770–8.
23. Humphrey RK, Beatle GM, Lopez AD, et al. Maintenance of pluripotency in human embryonic stem cells is STAT3 independent. Stem Cells. 2004;22:522–30.
24. Sumi T, Fujimoto Y, Nakatsuji N, et al. STAT3 is dispensable for maintenance of self-renewal in nonhuman primate embryonic stem cells. Stem Cells. 2004;22:861–72.
25. Kim SJ, Cheon SH, Yoo SJ, et al. Contribution of the PI3K/Akt/PKB signal pathway to maintenance of self-renewal in human embryonic stem cells. FEBS Lett. 2005;579:534–40.
26. Ishizuka T, Goshima H, Ozawa A, et al. Stimulation of α1-adrenoceptor or angiotensin type 1 receptor enhances DNA synthesis in human-induced pluripotent stem cells via Gq-coupled receptor-dependent signaling pathways. Eur J Pharmacol. 2013;714:202–9.
27. Massague J, Chen YG. Controlling TGF-beta signaling. Genes Dev. 2000;14:627–44.
28. Valdimarsdottir G, Mummery C. Functions of the TGFbeta superfamily in human embryonic stem cells. Apmis. 2005;113:773–89.
29. Beattie GM, Lopez AD, Bucay N, et al. Activin A maintains pluripotency of human embryonic stem cells in the absence of feeder layers. Stem cells. 2005;23:489–95.
30. James D, Levine AJ, Besser D, et al. TGFbeta/ activin/ nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. Development. 2005;132:1273–82.
31. Wang G, Zhang H, Zhao Y, et al. Noggin and bFGF cooperate to maintain the pluripotency of human embryonic stem cells in the absence of feeder layers. Biochem Biophys Res Commun. 2005;330:934–42.
32. Xu RH, Peck RM, Li DS, et al. Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. Nat Methods. 2005;2:185–90.
33. Zhang P, Li J, Tan Z, et al. Short-term BMP-4 treatment initiates mesoderm induction in human embryonic stem cells. Blood. 2008;111:1933–41.
34. Chadwick K, Wang L, Li L, et al. Cytokines and BMP-4 promote hematopoietic differentiation of human embryonic stem cells. Blood. 2003;102:906–15.
35. Klaus A, Sega Y, Taketo MM, et al. Distinct roles of Wnt/beta-catenin and Bmp signaling during early cardiogenesis. Proc Nat Acad Sci USA. 2007;104:18531–6.
36. Schulthesis TM, Burch JB, Lassar AB. A role for bone morphogenetic proteins in the induction of cardiac myogenesis. Genes Dev. 1997;11:451–62.
37. Gadue P, Huber TL, Paddison PJ, et al. Wnt and TGF-beta signaling are required for the induction of an in vitro model of primitive streak formation using embryonic stem cells. Proc Nat Acad Sci USA. 2006;103:16806–11.
38. Kattman SJ, Huber TL, Keller GM. Multipotent Flk-1+ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages. Dev Cell. 2006;11:723–32.

39. Kattman SJ, Witty AD, Gagliardi M, et al. Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. Cell Stem Cell. 2011;8:228–40.
40. Yang L, Soonpaa MH, Adler ED, et al. Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell- derived population. Nature. 2008;453:524–8.
41. Bai H, Gao Y, Arzigian M, et al. BMP4 regulates vascular progenitor development in human embryonic stem cells through a Smad-dependent pathway. J Cell Biochem. 2010;109:363–74.
42. Yan L, Jia Z, Cui J, et al. Beta-adrenergic signals regulate cardiac differentiation of mouse embryonic stem cells via mitogen-activated protein kinase pathways. Develop Growth Differ. 2011;53:772–9.
43. Chen Y, Shao JZ, Xiang LX, et al. Cyclic adenosine 3′,5′-monophosphate induces differentiation of mouse embryonic stem cells into cardiomyocytes. Cell Biol Int. 2006;30:301–7.
44. Drab M, Haller H, Bychkov R, et al. From totipotent embryonic stem cells to spontaneously contracting smooth muscle cells: A retinoic acid and db-cAMP in vitro differentiation model. FASEB J. 1997;11:905–15.
45. Ishizuka T, Goshima H, Ozawa A, et al. Involvement of β-adrenoceptors in the differentiation of human induced pluripotent stem cells into mesodermal progenitor cells. Eur J Pharmacol. 2014;740:28–34.
46. Ross SA, McCaffery PJ, Drager UC, et al. Retinoids in embryonal development. Physiol Rev. 2000;80:1021–54.
47. Shan ZY, Shen JL, Li QM, et al. pCREB is involved in neural induction of mouse embryonic stem cells by RA. Anat Rec. 2008;291:519–26.
48. Okada Y, Shimazaki T, Sobue G, et al. Retinoic-acid- concentration-dependent acquisition of neural cell identity during in vitro differentiation of mouse embryonic stem cells. Dev Biol. 2004;275:124–42.
49. Chrivia JC, Kwok RP, Lamb N, et al. Phosphorylated CREB binds specifically to the nuclear protein CBP. Nature. 1993;365:855–9.
50. Duman RS, Malberg J, Nakagawa S, et al. Neuronal plasticity and survival in mood disorders. Biol Psychiatry. 2000;48:732–9.
51. Di-Gregorio A, Sancho W, Stucky DW, et al. BMP signaling inhibits premature neural differentiation in the mouse embryo. Development. 2007;134:3359–69.
52. Kawasaki H, Mizuseki K, Nishikawa S, et al. Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity. Neuron. 2000;28:31–40.
53. Tropepe V, Hitoshi S, Sirard C, et al. Direct neural fate specification from embryonic stem cell: a primitive mammalian neural stem cell stage acquired through a default mechanism. Neuron. 2001;30:65–78.
54. Ying QL, Nichols J, Chambers I, et al. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. Cell. 2003;115:281–92.
55. Zhang K, Li L, Huang C, et al. Distinct functions of BMP4 during different stages of mouse ES cell neural commitment. Development. 2010;137:2095–105.
56. Chambers SM, Fasano CA, Papapetrou EP, et al. Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nat Biotechnol. 2009;27:275–80.
57. Smith JR, Vallier L, Lupo G, et al. Inhibition of activin/nodal signaling promotes specification of human embryonic stem cells into neuroectoderm. Dev Biol. 2008;313:107–17.
58. Matulka K, Lin HH, Hribkova H, et al. PTP1B is an effector of activin signaling and regulates neural specification of embryonic stem cells. Cell Stem Cell. 2013;13:706–19.
59. Deng W, Ohrocka M, Fischer I, et al. In vitro differentiation of human marrow stromal cells into early progenitors of neural cells by conditions that increase intracellular cyclic AMP. Biochem Biophys Res Commun. 2001;282:148–52.
60. Jori FP, Napolitano MA, Melone MA, et al. Molecular pathways involved in neural in vitro differentiation of marrow stromal stem cells. J Cell Biochem. 2005;94:645–55.

61. Wang TT, Tio M, Lee W, et al. Neural differentiation of mesenchymal-like stem cells from cord blood is mediated by PKA. Biochem Biophys Res Commun. 2007;357:1021–7.
62. Ishizuka T, Goshima H, Ozawa A, et al. β_1-adrenoceptor stimulation enhances the differentiation of mouse induced pluripotent stem cells into neural progenitor cells. Neurosci. Lett. 2012;525:60–5.
63. Banasr M, Hery M, Printemps R, et al. Serotonin-induced increases in adult cell proliferation and neurogenesis are mediated through different and common 5-HT receptor subtypes in the dentate gyrus and the subventricular zone. Neuropsychopharmacology. 2004;29:450–60.
64. Mahar I, Bambino FR, Mechawar N, et al. Stress, serotonin, and hippocampal neurogenesis in relation to depression and antidepressant effects. Neurosci Biobehav Rev. 2014;38:173–92.
65. Sahay A, Hen R. Adult hippocampal neurogenesis in depression. Nat Neurosci. 2007;10:1110–5.
66. Klempin F, Babu H, De Pietri Toneli D, et al. Oppositional effects of serotonin receptors 5-HT1a, 2, and 2c in the regulation of adult hippocampal neurogenesis. Front Mol Neurosci. 2010;3:1–11.
67. Zusso M, Debetto P, Guidolin D, et al. Fluoxetine-induced proliferation and differentiation of neural progenitor cells isolated from rat postnatal cerebellum. Biochem Pharmacol. 2008;76:391–403.
68. Ishizuka T, Goshima H, Ozawa A, et al. Stimulation of 5-HT4 receptor enhances differentiation of mouse induced pluripotent stem cells into neural progenitor cells. Clin Exp Pharmacol Physiol. 2014;41:345–50.
69. Reddy UR, Basu A, Bannerman P, et al. ZPK inhibits PKA induced transcriptional activation by CREB and blocks retinoic acid induced neuronal differentiation. Oncogene. 1999;18:4474–84.

Chapter 3
Regulation of microRNA Expression by Growth Factors in Tumor Development and Progression

Hiroshi Itoh, Sotai Kimura, and Seiji Naganuma

Abstract MicroRNAs (miRNAs) are a class of noncoding small RNAs (22–25 nucleotides) that regulate cell proliferation and various cellular functions by interfering with the translation of target messenger RNAs (mRNAs). Altered expression of miRNAs is found in various human malignancies, and indeed, we previously reported that the expression of miR-205 and miR-21 was altered in human head and neck squamous cell carcinoma (HNSCC), by miRNA microarray analysis. We also confirmed that the expression of miR-200c and miR-27b was directly regulated by hepatocyte growth factor (HGF) in HNSCC cell line, HSC3. These results suggest the significance of miRNAs as a key regulatory molecule for achieving various functions of growth factors. Altered miRNA expression might contribute enhanced progressive and invasive characteristics, such as epithelial-mesenchymal transition (EMT), of malignant tumors by regulating the translation of growth factor-induced functional molecules. There are a growing number of reports that describe the translational regulation of growth factors, their receptors, and intracellular signaling molecules by miRNAs in various tumors. However, less of the reports describe the regulation of miRNA expression by a growth factor itself. In this article, we review the relation of tumor development and progression by growth factors with miRNA expression, especially the regulation of miRNA expression by growth factors, and focus on the cooperative interactions of miRNAs, their mRNA targets, and growth factor signaling, in the context of tumor progression.

Keywords microRNA (miRNA) • growth factor • Hepatocyte growth factor (HGF) • Epidermal growth factor (EGF) • Post-transcriptional regulation

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H. Itoh, M.D., Ph.D. (✉) • S. Kimura, M.D., Ph.D.
Department of Molecular Pathology, Graduate School of Medicine,
Yamaguchi University, Ube, Japan
e-mail: hiroitoh@yamaguchi-u.ac.jp

S. Naganuma, M.D., Ph.D.
Department of Pathology, Kochi Medical School, Kochi University,
Nankoku, Kochi, Japan

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N. Shinomiya et al. (eds.), *Regulation of Signal Transduction in Human Cell Research*, Current Human Cell Research and Applications,
https://doi.org/10.1007/978-981-10-7296-3_3

3.1 Introduction

Tumor development and progression involve many cellular functions, such as cell proliferation, migration, invasion, and metastasis via blood and lymphatic vessels. These processes are tightly regulated by signal transduction cascades of growth factors (GFs), their receptors (GFRs), and intracellular signal transduction molecules (reviewed in [1]). Various GFs including epidermal growth factor (EGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), transforming growth factors (TGF)-β, vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) have been derived from human tumors. Their specific receptors, such as EGF receptor (EGFR) and HGF receptor, namely, MET, were also expressed in various human tumors, so that the tissue- or organ-specific expression of GFs and GFRs is responsible for cellular growth and functions mainly in autocrine or paracrine manner [1]. GFRs are similar single-pass transmembrane proteins having an intracellular tyrosine (called as receptor tyrosine kinase; RTK) for most of GFRs or serine/threonine kinase domain for TGF-β receptor [1, 2]. Of interest is that their roles in tumorigenesis are very similar with those in embryogenesis [3]. GFs regulate and maintain the extracellular matrix (ECM) with stromal cells including fibroblasts, myofibroblasts, and endothelial cells, as well as blood cells including various inflammatory cells both in tumorigenesis and embryogenesis.

MicroRNAs (miRNAs) are recently identified noncoding small RNAs (22–25 nucleotides) that regulate cell proliferation and various cellular functions by interfering with the translation of target messenger RNAs (mRNAs) (reviewed in [4]). In addition to modulate gene expression of GFs themselves, miRNAs also play regulatory roles in GF-induced signaling molecules, resulting in tumor development and progression [4, 5]. GFs are regulated by abundance of certain miRNAs and modulate the abundance of proteins necessary for GF-induced signaling cascades [6]. Altered expression of miRNAs is known to induce tumor growth and progression, and indeed, we previously reported that the expression of miR-205 and miR-21 was altered in human head and neck squamous cell carcinoma (HNSCC), by miRNA microarray analysis [7].

In this article, we review the relation of tumor development and progression by GFs with miRNA expression, especially the regulation of miRNA expression by GFs, and focus on the cooperative interactions of miRNAs, their mRNA targets and GF signaling, in the context of tumor progression. We also discuss the possibility of miRNAs as diagnostic, prognostic, and therapeutic markers for various tumors.

3.2 Signal Transduction and Functions of Growth Factors

GFs are compact polypeptides binding to their specific transmembrane receptors, most of them are RTKs [1, 2]. After binding of a GF to its specific receptor, the intracellular domain of GFR is phosphorylated, and the signal is simultaneously

transduced into the downstream signaling pathways, such as the mitogen-activated protein kinase (MAPK) pathway, the phosphatidylinositol 3-kinase (PI3K) pathway, phospholipase C-γ pathway, or transcription factors such as the signal transducers and activators of transcription (STATs) or SMAD proteins [1, 2]. These signal transduction cascades are essential for tumor development and progression, because the signaling molecules involved in these cascades can receive various oncogenic mutations in the initiated cells, namely, cancer stem cells, for clonal expansion of the tumor [8]. Indeed, oncogenic mutations are frequently seen in GFs and GFRs as well as protein kinase cascade molecules downstream of GFRs in various malignant tumors, (e.g., malignant melanoma (B-RAF), pancreatic cancer (RAS), breast cancer (ErbB-2/HER2), and brain tumor (EGFR)) [6]. GFs show various cellular functions mainly in paracrine manner in case of non-tumorous physiological condition such as embryogenesis and wound healing, but many cancer cells acquire the ability to synthesize GFs in addition to their specific receptors [9]. In such cases, cancer cells have constitutive autoactivation pathway in several distinct mechanisms. For example, overexpression of GFRs may enable cancer cells to become hyperresponsive to GFs, and indeed ErbB-2/HER2 gene amplification is frequently found in breast cancer [10]. Also, GFRs can get the ligand-independent signaling by specific gene mutations or deletions, and indeed EGFR is frequently mutated in brain tumors with EGF-independent autoactivation [11]. Similarly, the mutation of signal transducer molecules downstream of GFR such as RAS, which is mutated up to 25% of human cancers, may also show autonomic growth [12], and the effector molecule of TGF-β, namely, SMAD4/DPC4, is also mutated and inactivated in pancreatic cancer [13].

Tumor progression involves the following several steps: expand growth in primary site, disruption and penetration of basement membrane by cancer cells, invasion into adjacent tissues and enter the vascular or lymphatic systems (intravasation), departure from the bloodstream (extravasation), and subsequent colonization and regrowth in distant organs (metastasis) [14]. Thus, GFs and their signaling pathways play critical roles in this process. Cancer cells also show the morphological changes from the nest of epithelial cells into individual spindle-shaped mesenchymal-like cells, called as epithelial-mesenchymal transition (EMT) [14, 15]. Constitutive signaling of GFs via the tyrosine kinase domain may provide the second hits of tumor progression. TGF-β, HGF, and FGFs may induce EMT and enhanced invasive potential of cancer cells by upregulating various secreted proteases (e.g., the matrix metalloproteinases, MMP-2 and MMP-9) and downregulating protease inhibitors (e.g., the tissue inhibitors of metalloproteinases TIMPs) [16, 17]. At the same time, GFs also induce several molecular switches of adhesion: downregulation of the epithelial E-cadherin and upregulation of the mesenchymal N-cadherin [18]. Moreover, they also induce the expression of potent E-cadherin repressors (e.g., ZEB-2 and Twist) [18]. Generation of new vessels (neovascularization) is also critical for tumor growth, and angiogenic growth factors, such as VEGFs, FGFs, and TGF-β, play important roles in such phenomenon [19, 20]. VEGF antagonists are already used for the patients with colonic or renal cancer [21].

3.3 Biogenesis and Functions of microRNAs

As described briefly in the introduction, miRNAs are noncoding small RNAs (22–25 nucleotides) that regulate posttranscriptional gene expression by hybridizing to the 3′-untranslated regions (UTRs) and interfering translation of their target mRNAs [4]. One miRNA may regulate the expression of thousands of different mRNAs, while some different miRNAs may regulate one same mRNA. To date, 2588 miRNAs have been identified and listed in the miRBase database (release 21: June 2014), and their genes are located in both protein-coding and noncoding regions of all chromosomes except for the Y chromosome [22]. Approximately 30% of protein-coding genes are thought to be under translational control by miRNAs and to be involved in a variety of cellular processes, including the regulation of cellular differentiation, proliferation, and apoptosis [4, 23]. Under normal physiological conditions, individual miRNAs show strict tissue-specific and developmental stage-specific expression especially in embryogenesis [24]. Therefore, aberrant expression of miRNA induces various human tumors, and they are clearly classified by miRNA expression profiles [5].

As shown in Fig. 3.1, miRNAs are generated by finely regulated multistep processes [25–27]. Briefly, they are initially transcribed by RNA polymerase II as long

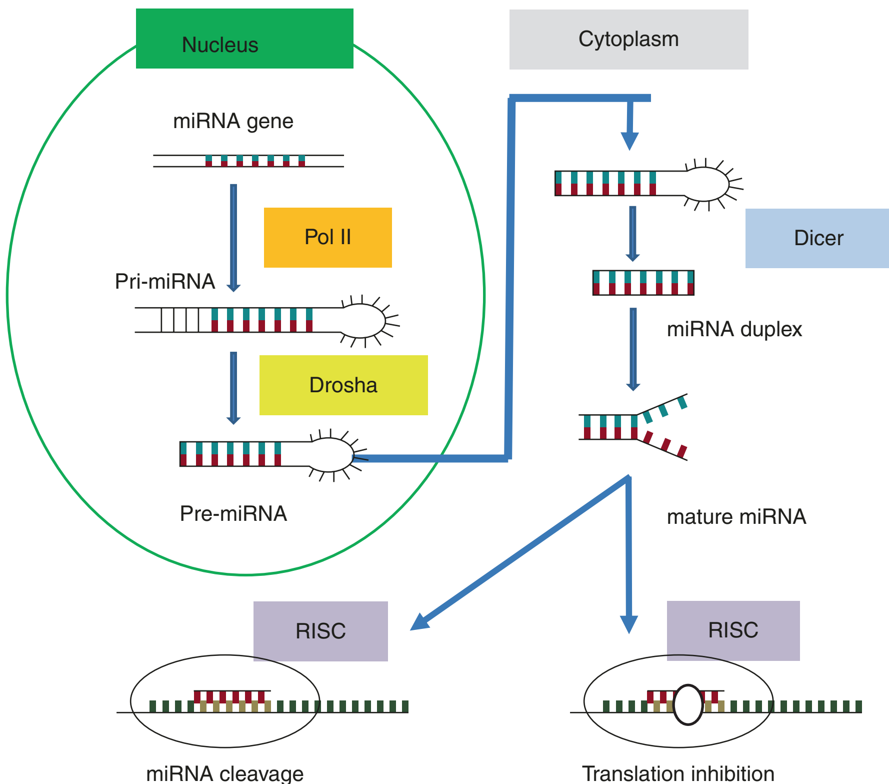

Fig. 3.1 Schematic representation of miRNA biosynthesis

primary transcripts with hairpin structures (pri-miRNAs) and are processed by Drosha to approximately 70-nucleotide precursor miRNAs (pre-miRNAs). After transport of the pre-miRNAs to the cytoplasm, mature single-stranded miRNAs are excised from the pre-miRNAs by RNase III enzyme, namely, Dicer, and then they are incorporated into the RNA-induced silencing complex (RISC). Finally, miRNAs regulate the gene expression binding through partial complementarity for the most part to the 3′-UTR of target mRNAs. When the nucleotide sequence of miRNA is perfectly matched to target miRNA sequence, the protein translation completely represses by cleaving and degrading the mRNA transcripts. On the other hand, a limited base-pair matching of miRNAs to target mRNA sequences leads to partial inhibition of the protein translation. Since miRNA is a negative regulator of gene expression targeting several hundreds of distinct mRNAs, a single miRNA can drastically affect various cellular processes involving cell proliferation, apoptosis, differentiation, and migration [27–30].

In the past few years, it has been reported that miRNAs have crucial roles in tumorigenesis (reviewed in [31]). First report of the relation of miRNA with malignant tumor described the deletion of the miR-15 and miR-16 loci in the majority of samples from patients with B-cell chronic lymphocytic leukemia (BCLL) [32]. Subsequently, altered miRNA expression has been reported in various tumors [5, 30]. In these tumors, it has been shown that miRNAs have key roles in tumor initiation, progression, and metastasis by using both loss-of-function and gain-of-function experiments in human-cultured tumor cells, transgenic mouse models, and knockout mouse models [33, 34]. Overexpression of the miR-17–92 cluster correlates with the expression of MYC and accelerates tumor development in a mouse model of B-cell lymphoma [35]. On the other hand, many other miRNAs function as tumor suppressors. For example, the let-7 family of miRNAs targets important oncogenes such as MYC, RAS family members (HRAS, KRAS, and NRAS), and high-mobility group AT-hook 2 (HMGA2) and suppresses the development of various tumors [36–38]. Thus, altered miRNA expression patterns in malignant tumors might be useful markers for diagnosis and prognosis of cancer patients as well as new therapeutic targets for various types of human cancer [5, 30, 39, 40]. Almost all cellular functions including cell proliferation, differentiation, survival, metabolism, genome stability, inflammation, invasion, and angiogenesis are under the control of miRNAs to affect tumor development and progression [31].

While each miRNA functions as either oncogene or tumor-suppressive gene, total miRNA expression level is generally suppressed in various tumor tissues compared with normal counterparts, [26]. Indeed, the downregulated expression of miRNA processing machinery components such as DROSHA and DICER (see Fig. 3.1) are observed in some malignant tumors including lung and ovarian cancers and neuroblastoma, and they are associated with advanced tumor stage and poor prognosis in the patients with these tumors [41–43]. This global suppression of miRNA expression was initially provided by the mouse model of lung cancer with gene deletion of the miRNA biogenesis pathway [44]. Thus, the miRNA biogenesis pathway can have an important role in cancer progression and might be impaired in various tumors. Indeed, it is recently reported that mutations and/or dysregulation of miRNA biogenesis pathway components affect pathophysiological relevance of the miRNA biogenesis machinery in human tumors [45–50].

3.4 Growth Factors and their Receptors Regulated by microRNAs

MicroRNAs can regulate the expression of GFs, GFRs, and their intracellular effector molecules, such as RAS and RAF, directly or indirectly [6, 23]. For example, EGFR family receptors can be regulated by multiple miRNAs and be extensively examined elsewhere [6]. Among many miRNAs regulating EGFR, miR-7 was identified as the first miRNA to downregulate EGFR directly in glioblastoma and lung and breast cancers [51, 52]. In addition to induce tumor-suppressive actions by regulating EGFR, miR-7 can also regulate the downstream signaling pathway at multiple sites. Indeed, EGFR downstream molecules, AKT and ERK1/2, are downregulated by miR-7 in several human cancer cell lines showing decreased invasiveness and cell cycle arrest [51, 52]. Similarly, miR-128 was the first miRNA identified as an upstream regulator of EGFR, and the loss of heterozygosity (LOH) of miR-128 gene was frequently detected in lung cancer, in correlation with patient survival and prognosis when treated with an EGFR-specific tyrosine kinase inhibitor [53]. Other miRNAs including miR-23b/27b, miR-133a, miR-133b, miR-146a, miR-146b-5p, miR-219-5p, miR-302b, and miR-608 can also regulate directly EGFR [54–62]. In addition, some miRNAs could target the effector molecules downstream of EGFR pathway. For example, miR-124, miR-147, and miR-193a-3p inhibit G1/S transition and cell proliferation by targeting EGFR-driven cell cycle proteins [63]. KRAS, BRAF, and MEK2 are also regulated by miR-143 and miR-145 in colorectal and prostatic cancers [64–66]. Both miR-27a (miR-27a-3p) and the complementary miR-27a* (miR-27a-5p) target EGFR as well as AKT and mammalian target of rapamycin (mTOR) in HNSCC cell lines [67]. Other members of the EGFR/ERBB family are also regulated by miRNAs in various human tumors [6]. HER2/ERBB2 was reported to be regulated by miR-552, miR-541, miR-193a-5p, miR-453, miR-134, miR-498, and miR-331-3p, using miRNA gain-of-function screens and two HER2-amplified cell lines [68]. miR-331-3p was also found to target HER2 directly in glioblastoma and prostate cancer cell lines [69, 70]. HER3/ERBB3 and the downstream signaling molecules were downregulated by miR-148b, miR-149, miR-326, and miR-520a-3p [71]. miR-125a and miR-125b target both HER2 and HER3 in breast cancer cells and consequently inhibit phosphorylation of ERK and AKT, while miR-193a-3p directly targets HER4/ERBB4 [72]. Overexpression of miR-193a-3p followed by repression of HER4 resulted in decreased proliferation, migration, invasion, and EMT, as well as increased apoptosis of lung cancer cells in xenograft tumor models [73, 74]. Also, miR-302 inhibited proliferation and invasion of esophageal squamous cell carcinoma cell line and induced apoptosis by targeting HER4 [75].

GFs and GFRs other than EGF and EGFR families are also regulated by certain miRNAs. TGF-β, an inflammation-related cytokine that functions in both tumor suppression and promotion, and its signaling pathway molecules are reported to be associated with many miRNAs [76]. Most members of the TGF-β signaling pathway are known to be targeted by one or more miRNAs [76–78]. Multiple binding sites for miR-744 is located in the proximal TGF-β1 3′-UTR, and miR-744

transfection inhibited endogenous TGF-β1 [79]. Suppression of miR-18a and miR-24 accounted for the derepression of two TGF-β1 processing factors, thrombospondin-1 (THBS1) and furin, respectively [80]. Ectopic expression of latent TGF-β1 reduces THBS1 protein expression and is associated with increased expression of let-7 and miR-18a [81]. On the other hand, downregulation of miR-30 or miR-200 upregulates TGF-βR I and Smad2 to induce the EMT and invasive potential of anaplastic thyroid carcinomas [82], and miR-128a promotes letrozole resistance by targeting TGF-βR I in breast cancer cells [83]. To date, many miRNAs targeting TGFβ, TGF-βR, and their downstream effector molecules have been identified [76–78].

The expression of HGF and its specific receptor MET pathway is also regulated by various miRNAs. Direct targeting of HGF by miR-16 regulates proliferation and migration in gastric cancer cells [84], and miR-206 inhibits HGF-induced EMT and angiogenesis in non-small cell lung cancer via MET/PI3K/AKT/mTOR pathway [85]. miR-26a and miR-198 suppress angiogenesis and inhibit migration and invasion of hepatocellular carcinoma cells by targeting HGF and MET signaling pathway, respectively [86, 87]. miR-34 family including miR-34a, miR-34b, and miR-34c negatively regulates MET and suppresses tumor growth and metastasis [88]. Many other miRNAs are reported to be involved in the expression of HGF/MET pathway [89].

As for other GFs and GFRs, IGF-1 signaling pathway regulates critical biological processes including development, homeostasis, and aging, and the expression is regulated by several miRNAs involving miR-1, miR-7, miR-99a, miR-145, miR-182, miR-223, and miR-320 [90]. FGF2 inhibits EMT through miR-20a-mediated repression of canonical TGF-β signaling [91]. miR-212 downregulation contributes to the protective effect of exercise against nonalcoholic fatty liver, and miR-577 inhibits pancreatic β-cell function and survival by targeting FGF-21 [92, 93]. microRNA-9 regulates cardiac fibrosis by targeting PDGFR-β in rats [94], and some other miRNAs regulate angiogenesis by targeting VEGF [95, 96]. In this way, there are a growing number of the reports describing the regulation of GFs, GFRs, and their intracellular effector molecules by miRNAs, and the list, although not all, is shown in Table 3.1.

Table 3.1 MicroRNAs that target various GFs, GFRs, and their intracellular effectors

miRNAs	Target GF-related genes	Regulation	Ref.
Let-7	RAS	Down	[4]
miR-1	Met, PIK3CA, IGF	Down	[89] [90]
miR-7	EGFR, IGFR	Down	[51] [52] [90]
miR-9	PDGFR	Down	[94]
miR-16	HGF	Down	[84]
miR-18	TGFβ	Down	[81]
miR-20a	TGFβR2, FGF	Up	[76] [91]
miR-21	Met, EGFR, TGFβR2	Up	[76] [89]
miR-23b	EGFR	Down	[54]

(continued)

Table 3.1 (continued)

miRNAs	Target GF-related genes	Regulation	Ref.
miR-24	TGFβ	Down	[81]
miR-26a	HGF	Down	[86]
miR-27a/b	Met, EGFR, MAP2K4	Down	[54] [64] [89]
miR-30b/c	Met, EGFR	Up	[89]
	TGFβ, PDGFR	Down	[82]
miR-34 family	Met, PDGFR, MAP2K1	Down	[88]
miR-99a	IGFR	Down	[90]
miR-103/203	Met, EGFR	Down	[89]
miR-106a	TGFβR2	Up	[76]
miR-124	EGFR	Down	[63]
miR-125a/b	HER2, HER3	Down	[72]
miR-126	VEGFR	Down	[95]
miR-128	EGFR, TGFβR	Down	[53] [83]
miR-130	Met, EGFR	Down	[89]
miR-133a/b	EGFR, TGFβ	Down	[55] [56] [57]
miR-134	Met, EGFR, PDGFR, HER2	Down	[68]
miR-141	MET	Up	[89]
miR-143	EGFR	Down	[64] [65] [66]
miR-145	EGFR, IGF	Down	[64] [65] [66] [90]
miR-146a/b	EGFR	Down	[58] [59]
miR-147	EGFR	Down	[58] [59]
miR-148b	HER3	Down	[71]
miR-149	HER3	Down	[71]
miR-182	Met, IGFR	Down	[89] [90]
miR-193a-3p	EGFR, HER4	Down	[63] [73] [74]
miR-193a-5p	HER2	Down	[68]
miR-199a	Met, ERK2	Down	[89]
miR-200 family	TGFβ, TGFβR	Down	[32]
	MET	Up	[76]
miR-206	HGF	Down	[85]
miR-212	FGF	Up	[82]
miR-219-5p	EGFR	Down	[60]
miR-221/222	Met, EGFR, kit, PDGFR	Up	[89] [96]
miR-223	IGFR	Down	[90]
miR-302b	EGFR, HER4	Down	[61] [75]
miR-320	IGF-1	Down	[90] [96]
miR-326	HER3	Down	[71]
miR-331-3p	HER2	Down	[68] [69] [70]
miR-340	MET	Down	[89]
miR-409-3p	MET	Down	[89]
miR-449a	Met,	Down	[89]
miR-453	HER2	Down	[68]
miR-497	VEGFR	Down	[95]

Table 3.1 (continued)

miRNAs	Target GF-related genes	Regulation	Ref.
miR-498	HER2	Down	[68]
miR-503	VEGFR, FGF	Down	[95]
miR-520a-3p	HER2, HER3	Down	[68] [71]
miR-541	HER2	Down	[62]
miR-552	HER2	Down	[62]
miR-577	FGF	Down	[93]
miR-599	TGFβ	Down	[76]
miR-608	EGFR	Down	[62]
miR-613	MET	Down	[89]
miR-744	TGFβ	Down	[79]
miR-7515	Met, AKT, ERK1/2	Down	[89]

3.5 microRNAs Regulated by Hepatocyte Growth Factor (HGF)

While many miRNAs directly regulate the expression of GFs, GFRs, and their intracellular effector molecules, the expression of miRNA is also controlled or influenced by GFs, conversely. Several studies reported that the expression profiles of miRNAs were drastically changed by the stimulation of cultured cells with specific growth factors. Among these GFs affecting the expression of miRNA, we focused on HGF, a multifunctional pleiotropic growth factor that acts as mitogen, motogen, and/or morphogen in a variety of cells including epithelial and endothelial cells [97–99]. It is also known to be a scatter factor (SF) [97] and involved in tumor-stromal interactions, angiogenesis, and EMT [100, 101]. MET is a specific receptor tyrosine kinase for HGF and upregulated in various tumors. HGF/SF and MET signaling is transduced to the nucleus and induces the expression of genes for the progressive and invasive characteristics of various tumors in paracrine or autocrine manner [98, 99, 102]. Indeed, HGF/SF is reported to promote cell migration and angiogenesis [103], to upregulate the expression of proangiogenic cytokines IL-8 and VEGF through the activation of MEK and PI3K signaling pathways [104], and to induce the expression of MMPs through the upregulation of the transcription factor E1AF [105]. However, although HGF/SF is a multifunctional growth factor, how HGF/SF and MET signaling induces the expression of each specific downstream functional gene have not yet been elucidated in detail. We recently demonstrated that several miRNAs affecting the translation of HGF-induced downstream functional molecules could also be regulated by HGF itself [106]. In this paper, we examined the expression of miRNAs that regulate cell proliferation and functions by interfering the translation of target mRNAs, with or without HGF stimulation in HNSCC cell line HSC3. Among

several miRNAs that the expression was altered after HGF stimulation, we focused on miR-200c and miR-27b, both of which were drastically downregulated after HGF stimulation, because of their unique target mRNAs affecting HGF-induced functional molecules. One of target mRNAs for miR-200c is a transcriptional regulator of E-cadherin, ZEB1 [107, 108]. In our study, ZRB1 mRNA was upregulated 6 h after HGF stimulation, and its downstream functional molecule E-cadherin mRNA was downregulated 12 h after HGF stimulation. Therefore, through the downregulation of miR-200c, HGF might have an important role for EMT and cancer cell migration and scattering. On the other hand, one of target mRNAs for miR-27b has recently been reported to be ST14/matriptase that is a cell surface proteinase for ECM degradation and HGF activation [109]. In our study, ST14/matriptase mRNA expression was not drastically altered after HGF stimulation, but its translated protein was drastically upregulated by western blotting. Thus, translational suppression of ST14/matriptase mRNA might be interrupted by downregulated miR-27b without destruction of ST14/matriptase mRNA. ST14/matriptase might inhibit cell growth as well as cell invasion and migration as a tumor suppressor [109]. However, other reports showed that ST14/matriptase is overexpressed in 100% of primary squamous cervical tumors, and 40% of cervical adenocarcinoma cell lines [110] participates in mammary epithelial cell growth and morphogenesis through HGF activation [111] and enhances cell adhesion in colorectal cancer cells and squamous cell carcinogenesis [112, 113]. Thus, ST14/matriptase may have different functions in different cancer cell types. In addition, HGF might be autoactivated by upregulated ST14/matriptase through downregulation of miR-27b after HGF simulation. Taken together, miR-200c and miR-27b downregulated after HGF stimulation might play an important role for EMT mediated by ZEB1/E-cadherin and tumor invasion and HGF autoactivation mediated by ST14/matriptase, respectively. Altered expression of miRNAs directly regulated by HGF might contribute enhanced progressive and invasive characteristics, by regulating the translation of HGF-induced downstream functional molecules.

Several miRNAs other than miR-200c and miR-27b were also downregulated after HGF stimulation in miRNA microarray analysis (106, see Table 3.2). Let-7a and miR-16, well-known tumor-suppressive miRNAs described above [114–116], were markedly downregulated after HGF stimulation. Let-7 family miRNAs including let-7a are downregulated in various human malignancies and negatively regulate RAS oncogenes that are most frequently activated in many cancers [37, 114, 117]. Downregulation of let-7 family miRNAs is also shown to cause radioresistance and results in poor prognosis [118]. On the other hand, the expression of miR-16 induces apoptosis by targeting anti-apoptotic gene BCL2 and is downregulated in many human malignancies including hematological cancer [32, 114, 119]. It has been recently reported that suppression of MYC oncogene by miR-23a/b enhances mitochondrial glutaminase expression

Table 3.2 Differential expression of miRNAs after HGF stimulation in HNSCC cell line HSC3 cells by statistical analysis of miRNA microarray (see ref. 106).

	miRNA	Fold increases	Predicted target gene	Ref.
Downregulation (<0.5 fold)				
1	Let-7a	0.16	RAS, myc	[37]
2	miR-23a	0.20	myc	[120]
3	miR-205	0.25	MED1	[121]
4	miR-200c	0.25	ZEB1	[107]
5	miR-27a	0.36	ZBTB1	[122]
6	miR-27b	0.44	ST14/Matriptase	[109]
7	miR-16	0.47	BCL2	[32]
Upregulation (>3.0-fold)				
1	miR-200a	3.89	ZEB2, CTNNB1	[107]
2	miR-141	11.90	ZEB2	[108]

Each value was shown in net intensity after quantile normalization

and glutamine metabolism [120]. On the other hand, target mRNAs of miR-205 and miR-27a were reported to be a transcriptional coactivator MED1 and a transcription factor ZBTB1, respectively, although their functions and roles in human tumors are still limited [121–123]. miR-205 might also regulate EMT by targeting ZEB1 and SIP1 in collaboration with miR-200 family miRNAs including miR-200c and target HER3 oncogene in human breast cancer [107, 108, 124]. It has been recently reported that low-level expression of miR-205 is a prognostic marker of HNSCC and that downregulation of microRNAs of the miR-200 family and miR-205 followed by an altered expression of classic and desmosomal cadherins were observed in spindle cell carcinoma of the head and neck with EMT [125, 126]. Therefore, in addition to miR-200c, downregulation of miR-205 after HGF stimulation might also play an important role in tumor progression in association with EMT. Conversely, upregulated miRNAs after HGF stimulation were limited. In our study, miR-200a and miR-141 were significantly upregulated, both of which are considered to regulate the translation of ZEB2 mRNA [107]. ZEB2 is a transcription factor that regulates the transcription of EMT-associated genes in cooperation with ZEB1 [107, 108]. Thus, upregulated miR-200a and miR-141 might be coordinated with downregulated miR-200c and miR-205 and then play an important role in the translational regulation of EMT-associated genes after HGF stimulation.

In this way, HGF signaling cascades involve in tumor progression and invasion through MET receptor tyrosine kinase in paracrine or autocrine manner by directly regulating the transcription of downstream functional molecules as well as by interfering the translation of these molecules through miRNAs regulated by HGF itself. The schematic representation of possible roles of miRNAs regulated by HGF is shown in Fig. 3.2.

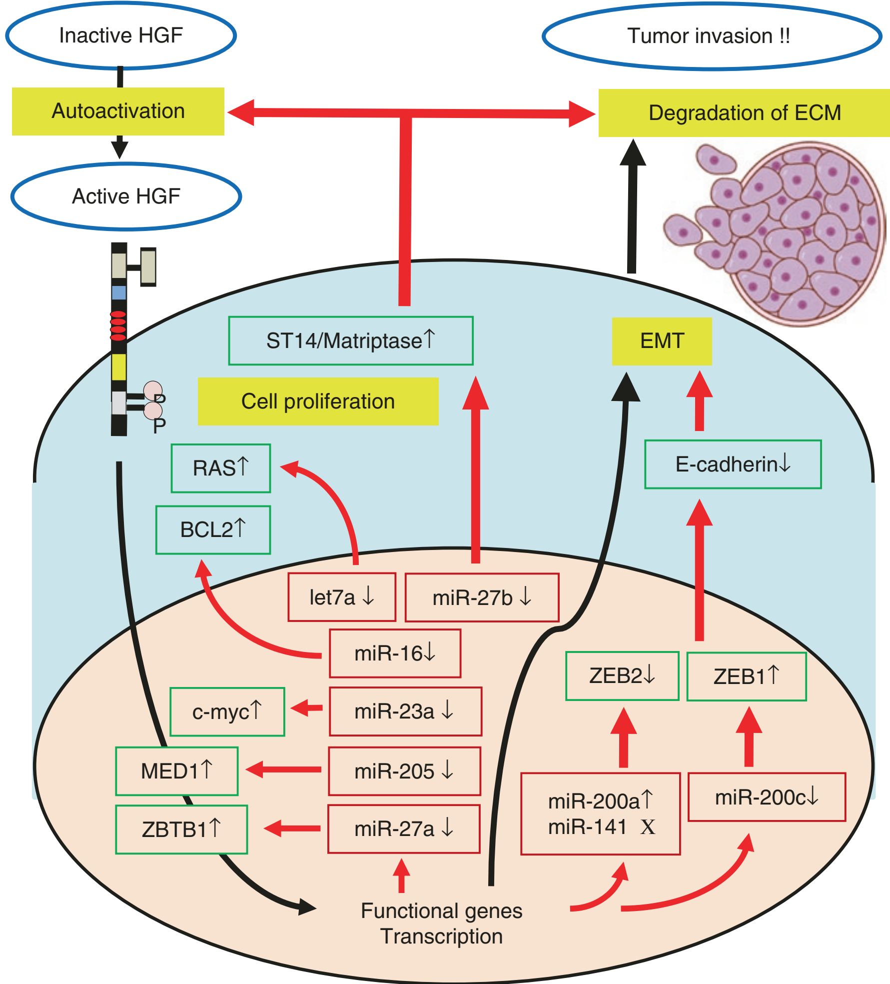

Fig. 3.2 Schematic representation of predicted signaling pathways and functions of miRNAs regulated by HGF

3.6 microRNAs Regulated by Other Growth Factors

GFs other than HGF also regulate the expression of miRNA. Among them, EGF is most widely examined by Kedmi M et al. and other investigators. They showed that dynamic and coordinated changes in expression of miRNAs were identified in normal mammary epithelial cells following stimulation with EGF [127, 128]. In this study, both up- and downregulation of distinct groups of miRNAs were observed in less than 60 min after EGF stimulation. A group of 23 miRNAs immediately downregulated by EGF stimulation were overrepresented among miRNAs that showed

lower expression in breast cancer tissue compared to the surrounding normal tissue in the same patient [127]. Since breast cancer cell line MCF10A cells migrated in response to EGF stimulation [129, 130], the migratory response of these cells could be controlled by both up- and downregulated miRNAs. Among miRNAs immediately upregulated by EGF stimulation, miR-15b was expressed significantly higher in different breast cancer subtypes. Metastasis suppressor 1 (MTSS1), a lipid-binder cytoskeletal protein and a novel target of miR-15b, was downregulated after EGF stimulation and affected the migration and invasion ability of normal and cancer cells, and indeed, the expression was lost in some advanced cancers [128]. Conversely, among the miRNAs immediately downregulated by EGF stimulation, miR-191 targets one of the immediate early genes (IEGs) called EGR1 and suppresses cell migration [128]. Although many other miRNAs immediately downregulated by EGF stimulation can also target IEGs, such as FOS and JUN proto-oncogenes, they inhibit the expression of IEGs in the steady state without EGF stimulation. However, these miRNAs are immediately downregulated by EGF stimulation, and the IEGs are rapidly upregulated. Interestingly, miR-155, one of the miRNAs immediately downregulated by EGF stimulation, directly targets FOS, but the oncogenic viral form of c-FOS, namely, v-FOS, harbors a shorter 3′-UTR than the c-FOS 3′-UTR, which does not include miR-155's target sequence. Thus, the transcript of v-FOS is not inhibited by miR-155 and is thought to show constitutional oncogenic ability. Also, other predicted targets of the miRNAs immediately downregulated by EGF stimulation involved in molecular functions that relate to EGF signaling, such as cellular development, proliferation, cell morphology, cell death, and cell-to-cell signaling and interaction [131]. On the other hand, miR-31, miR-181b, and miR-222 were upregulated in oral cancer cells with EGF stimulation mediated by AKT and C/EBPβ signaling pathway [132]. Increased expression of miR-31 was also observed in EGF-stimulated mammary cells and directly targets synaptojanin 2 (SYNJ2), a lipid phosphatase [128]. miR-31 downregulates transiently SYNJ2 expression and then upregulates SYNJ2 again back to baseline expression level. The expression of SYNJ2 was negatively correlated with miR-31 expression and associated with poor prognosis in patients with breast or brain cancer [133]. Overexpression of SYNJ2 enhanced tumor growth and metastasis in mice and increased formation of invadopodia and lamellipodia, actin-filled cellular extensions involved in invasion and migration, respectively [133]. In addition to the immediate response, the delayed response to EGF stimulation (3–12 h post stimulation) is also seen in miRNAs targeting both apoptotic and anti-apoptotic genes. Especially, miR-134, miR-145, miR-146b, miR-432, and miR-494 had many apoptotic and anti-apoptotic targets including the interferon pathway [134]. miR-221/222 is identified as a regulator of apoptosis and is induced by not only EGFR but also MET [135]. The miR-30 family is induced by SRC inhibitors and downregulated by oncogenic GF signaling molecules such as EGF and HGF as well as MAPK-regulated transcription factor, ERG, in association with EMT [136]. Therefore, several miRNAs induced by GFs seem to act cooperatively to support cellular proliferation and functions of various tumors.

Aberrant expression of some miRNAs controlled by GF signaling pathways implicates in the pathogenesis of not only cancers but also other diseases. miR-21 is a well-known oncogenic miRNA and is upregulated in nearly all tumor samples [137], by targeting the transcripts of tumor suppressor genes such as programmed cell death protein 4 (PDCD4), tropomycin1 (TPM1), and phosphatase and tensin homolog (PTEN) [138–140]. Since TGF-β signaling is often associated with various tumor types, increased expression of miR-21 is, in part, due to aberrant activation of TGF-β signal [141]. Similarly, upregulation of miR-21 can be initiated by cardiac stress, resulting in cardiac hypertrophy or fibrosis in a MAPK/ERK pathway-dependent manner [137]. On the other hand, let-7 family of miRNAs is well known as a tumor-suppressive miRNA and targets several oncogenes including MYC and RAS family of oncogenes and high-mobility group A2 (HMGA2) [37, 142–143]. Biogenesis of let-7 is inhibited by ERK1/2-mediated pathway, and the expression is often lower in tumor samples than in normal tissues, unlike miR-21 [144] (Fig. 3.3).

The interaction between TGF-β signaling and miRNAs has also been investigated extensively, and TGF-β pathway signals could either inhibit or promote miRNA maturation [145, 146]. Davis et al. showed that TGF-β treatment resulted in the upregulation of pre-miRNAs and mature miRNAs, but not that of pri-miRNAs [147]. Smad proteins have also been shown to control the transcription of miRNA-coding genes by binding to miRNA promoter genes under control of TGF-β [146, 148, 149]. miR-21, miR-181, miR-494, miR-455-5p, and miR-10 are prominently upregulated by TGF-β signaling [76–78]. A meta-analysis revealed that high miR-21 levels are related to poor overall survival of various human tumors [150]. The expression of miR-21 induced by TGF-β is markedly higher in cancer cells and contributes to chemoresistance in breast cancer cells by targeting the MutS homolog 2 (MSH2) [151]. Wang et al. demonstrated that miR-181b mediated by TGF-β promotes the generation of hepatocellular carcinoma by targeting the tissue inhibitor of metalloprotease 3 (TIMP3) [152]. Similarly, Liu et al. showed that miR-494 upregulated by TGF-β is required for the accumulation and activity of MDSCs via targeting of PTEN [153]. The level of miR-10b expression upregulated by TGF-β signaling pathway correlates with clinical progression of primary breast carcinomas [154, 155]. Upregulation of miR-455-5p by the TGF-β-SMAD signaling axis promotes the proliferation of oral squamous cancer cells by targeting UBE2B [156]. In this way, TGF-β upregulates many miRNAs including miR-27a, miR-183, miR-182, miR-155, and miR-451 [76–78], but miR-200, miR-34a, miR-203, miR-584, and miR-450b-5p are conversely downregulated by the TGF-β/Smad signaling pathway [76–78]. Gregory et al. reported that all five members of the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) are markedly downregulated in cells showing EMT in response to TGF-β [107]. Yang et al. demonstrated that elevated TGF-β activity associated with the persistent presence of hepatitis B virus in liver tissues suppresses the expression of miR-34a, leading to enhanced production of the chemokine CCL22, which recruits regulatory T cells to facilitate immune escape [157]. Xu et al. showed that miR-203 is downregulated in renal cell carcinoma, and FGF2 is a direct target of miR-203. Ding et al. demonstrated that TGF-β represses the expression of miR-203 to promote EMT and tumor metastasis

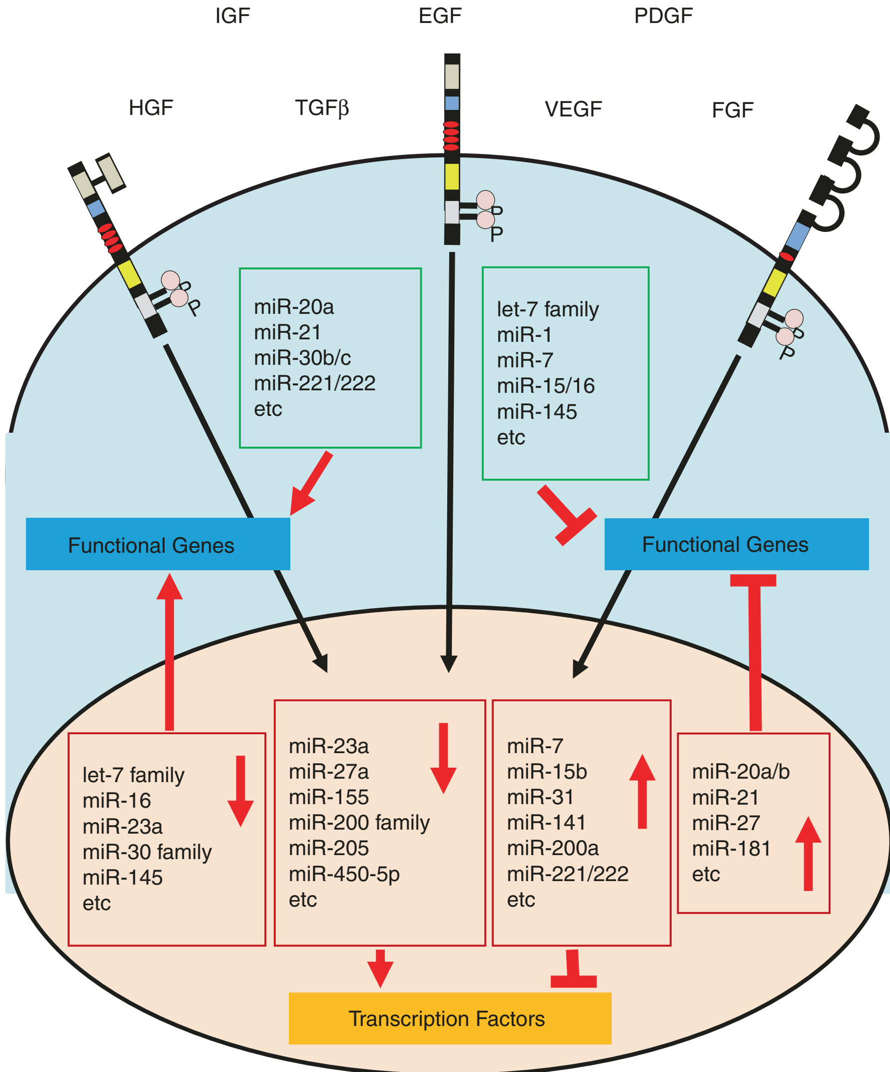

Fig. 3.3 Schematic representation of predicted signaling pathways and functions of miRNAs regulating or regulated by various growth factors

[158, 159]. Similarly, miR-584 is reported to be a potential tumor suppressor, and the expression is negatively regulated by TGF-β in a number of breast cancer cells [160]. TGF-β1 was shown to exert its function by suppressing miR-450b-5p, which significantly inhibited the growth of rhabdomyosarcoma and promoted the expression of MyoD [161]. As for angiogenetic GFs, FGF regulates TGF-β signaling and EMT via control of let-7 miRNA expression [162]. PDGF-BB enhances the proliferation of cells in human orbital fibroblasts by suppressing PDCD4 expression via

upregulation of microRNA-21 [163] and miR-15b induced by PDGF signaling is required for vascular smooth muscle cell proliferation [164]. Table 3.2 shows the list of miRNAs regulated by various GFs, although not all.

3.7 Growth Factor and microRNA Crosstalk Network

Two-way interactive regulation of GFs and miRNAs in various tumors may result in feedback regulatory loops in which a single miRNA is targeting a specific pathway and is also regulated by the same pathway at the same time. For example, several miRNAs directly targeting EGFR are also regulated by EGFR signaling pathway. Among one of these miRNAs, miR-34a is immediately upregulated by EGF stimulation, but it is also directly regulating EGFR [127, 128]. Indeed, miR-34a acts as a tumor suppressor in the development of chordoma through this complex regulation [62]. miR-7 is a well-established regulator of EGFR, but it was also shown to be regulated by EGFR signaling pathway. Activation of EGFR in lung cancer cells can stimulate miR-7 expression in an ERK-dependent manner, suggesting that EGFR induces miR-7 expression via the RAS-ERK pathway [165]. Feedback loops of specific miRNAs are also found in the different components of the EGFR pathway. For example, miR-143 and miR-145 regulate the EGFR pathway genes KRAS, BRAF, and MEK2 [64, 65], but EGFR signals also downregulate these tumor suppressor miRNAs through ERK1/2 in a murine model of colon cancer and lung cancer cells [166, 167]. Also, EGFR regulates the expression of miRNAs targeting other molecules of the same signaling pathway. The expression level of miR-21 is regulated by EGFR via the activation of beta-catenin and AP-1 and is suppressed by the EGFR inhibitor, AG1478, suggesting that the EGFR can also regulate miR-21 expression [168, 169]. On the other hand, miR-21 regulates EGFR and AKT signaling through VHL/beta-catenin and the PPARα/AP-1axis [168]. Taken together, these GF and miRNA crosstalk networks are thought to play an important role in cellular proliferation and various cellular functions involved in tumor development and progression (Table 3.3).

3.8 Concluding Remarks

GF signaling is essential for tumor development and progression and, therefore, is often targeted by antitumor drugs [170]. Since aberrant expression of miRNA is extensively found in cancer and the profiling of miRNAs can be detected in tumor samples as well as in patient fluids, miRNA is thought to be very useful as diagnosis, prognosis, and therapeutic markers [40, 171–173]. For example, as a first reported case, reduced expression of let-7 in human lung tumors is associated with shortened postoperative survival [174]. Because miRNAs are highly stable in various patient samples including tissue and blood samples, the use of let-7 and other

Table 3.3 MicroRNAs regulated by various GFs

miRNAs	Regulating GFs	Target genes	Ref.
Downregulation			
Let-7 family	HGF, FGF, TGFβ	RAS, myc	[106]
miR-16	HGF	BCL2	[106]
miR-23a	HGF	myc	[106]
miR-24	TGFβ	TPIM	[76]
miR-27a	HGF	ZBTB1	[106]
miR-27b	HGF, TGFβ	ST14/Matriptase	[106]
miR-30 family	EGF, HGF	ERG	[6]
miR-34a	TGFβ	CCL22	[157]
miR-142-3p	TGFβ	Unknown	[76]
miR-145	EGF	ERK1/2	[6]
miR-155	EGF, TGFβ	FOS	[76] [131]
miR-191	EGF	EGR1	[76]
miR-200 family	HGF, TGFβ	ZEB1	[106]
miR-203	TGFβ	FGF	[158] [159]
miR-205	HGF, TGFβ	MED1	[106]
miR-584	TGFβ	PHACTR1	[160]
miR-450-5p	TGFβ	ENOX2, PAX9	[161]
Upregulation			
miR-7	EGF	ETS2	[128]
miR-15b	EGF, PDGF	MTSS1	[128] [164]
miR-20a/b	EGF, FGF	TGFβ	[128]
miR-21	TGFβ, PDGF	MSH2	[150] [151]
miR-27	TGFβ	MAPK	[76]
miR-31	EGF	SYNJ2	[128]
miR-141	HGF	ZEB2	[106]
miR-146a	EGF, TGFβ	CYLD	[76]
miR-181b	EGF, TGFβ	AKT, CEBPβ, TIMP3	[132] [152]
miR-200a	HGF	ZEB2, CTNNB1	[106]
miR-221/222	EGF, HGF	AKT, CEBPβ	[132]
miR-455-5p	TGFβ	UBE2B	[156]
miR-494	TGFβ	PTEN	[153]

miRNAs as various biomarkers has been facilitated [171, 175]. Furthermore, the expression of miRNAs has been shown to be highly tissue specific and so that miRNA profiling might be able to determine the developmental origins of specific tumors [40, 176, 177]. In past few years, the global expression pattern of miRNAs has been used to classify the tumors of various organs [178–184]. Indeed, a miRNA microarray analysis identifies the primary origin of different types of tumors [175, 185]. In addition, Nair et al. reported that the changes of miRNAs most frequently associated with poor outcome were downregulation of let-7 and upregulation of miR-21 [186]. In the context of GF signaling, relative abundance of subsets of

EGF-regulated miRNAs in breast cancer models have been shown to correlate with the abundance of miRNAs in breast cancer patients [127, 128]. Thus, miRNAs could likely become major diagnostic and prognostic markers in the near future, and the crosstalk regulation of miRNAs and GF signals is expected to control patient response to therapeutic interventions, such as monoclonal antibodies. Resolving miRNA and GF crosstalk networks and understanding their functional roles could improve the future cancer treatments to avoid the therapeutic resistance in various cancer patients.

Conflicts of Interest The authors declare no conflict of interest.

References

1. Witsch E, Sela M, Yarden Y. Roles for growth factors in cancer progression. Physiology (Bethesda). 2010;25:85–101. https://doi.org/10.1152/physiol.00045.2009.
2. Yarden Y, Ullrich A. Growth factor receptor tyrosine kinases. Annu Rev Biochem. 1988;57:443–78.
3. Moustakas A, Heldin CH. Signaling networks guiding epithelial-mesenchymal transitions during embryogenesis and cancer progression. Cancer Sci. 2007;98:1512–20.
4. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116:281–97.
5. Calin GA, Groce CM. MicroRNA signatures in human cancers. Nat Rev Cancer. 2006;6:857–66.
6. Kedmi M, Sas-Chen A, Yarden Y. MicroRNAs and growth factors: an alliance propelling tumor progression. J Clin Med. 2015;4:1578–99. https://doi.org/10.3390/jcm4081578.
7. Kimura S, Naganuma S, Susuki D, et al. Expression of microRNAs in squamous cell carcinoma of human head and neck and the esophagus: miR-205 and miR-21 are specific markers for HNSCC and ESCC. Oncol Rep. 2010;23:1625–33.
8. Futreal PA, Coin L, Marshall M, et al. A census of human cancer genes. Nat Rev Cancer. 2004;4:177–83. https://doi.org/10.1038/nrc1299.
9. Sporn MB, Todaro GJ. Autocrine secretion and malignant transformation of cells. N Engl J Med. 1980;303:878–80.
10. Di Fiore PP, Pierce JH, Kraus MH, et al. erbB-2 is a potent oncogene when overexpressed in NIH/3T3 cells. Science. 1987;237:178–82.
11. Huang HS, Nagane M, Klingbeil CK, et al. The enhanced tumorigenic activity of a mutant epidermal growth factor receptor common in human cancers is mediated by threshold levels of constitutive tyrosine phosphorylation and unattenuated signaling. J Biol Chem. 1997;272:2927–35.
12. Shrestha G, MacNeil SM, McQuerry JA, et al. The value of genomics in dissecting the RAS-network and in guiding therapeutics for RAS-driven cancers. Semin Cell Dev Biol. 2016;58:108–17. https://doi.org/10.1016/j.semcdb.2016.06.012.
13. Chen WB, Lenschow W, Tiede K, et al. Smad4/DPC4-dependent regulation of biglycan gene expression by transforming growth factor-beta in pancreatic tumor cells. J Biol Chem. 2002;277:36118–28.
14. Yilmaz M, Christofori G. EMT, the cytoskeleton, and cancer cell invasion. Cancer Metastasis Rev. 2009;28:15–33.
15. Yeung KT, Yang J. Epithelial-mesenchymal transition in tumor metastasis. Mol Oncol. 2017;11:28–39. https://doi.org/10.1002/1878-0261.12017.

16. Stetler-Stevenson WG. Matrix metalloproteinases in angiogenesis: a moving target for therapeutic intervention. J Clin Invest. 1999;103:1237–41.
17. Werb Z. ECM and cell surface proteolysis: regulating cellular ecology. Cell. 1997;91:439–42.
18. Jiang WG, Sanders AJ, Katoh M, et al. Tissue invasion and metastasis: molecular, biological and clinical perspectives. Semin Cancer Biol. 2015;35(Suppl):S244–75. https://doi.org/10.1016/j.semcancer.2015.03.008.
19. Rak J, Filmus J, Finkenzeller G, et al. Oncogenes as inducers of tumor angiogenesis. Cancer Metastasis Rev. 1995;14:263–77.
20. Bikfalvi A. Significance of angiogenesis in tumour progression and metastasis. Eur J Cancer. 1995;31A:1101–4.
21. Lin Z, Zhang Q, Luo W. Angiogenesis inhibitors as therapeutic agents in cancer: challenges and future directions. Eur J Pharmacol. 2016;793:76–81. https://doi.org/10.1016/j.ejphar.2016.10.039.
22. Ghorai A, Ghosh U. miRNA gene counts in chromosomes vary widely in a species and biogenesis of miRNA largely depends on transcription or post-transcriptional processing of coding genes. Front Genet. 2014;5:100. https://doi.org/10.3389/fgene.2014.00100.
23. Donzelli S, Cioce M, Muti P, et al. MicroRNAs: non-coding fine tuners of receptor tyrosine kinase signalling in cancer. Semin Cell Dev Biol. 2016;50:133–42. https://doi.org/10.1016/j.semcdb.2015.12.020.
24. Ivey KN, Srivastava D. microRNAs as developmental regulators. Cold Spring Harb Perspect Biol. 2015;7:a008144. https://doi.org/10.1101/cshperspect.a008144.
25. Davis-Dusenbery BN, Hata A. Mechanisms of control of microRNA biogenesis. J Biochem. 2010;148:381–92. https://doi.org/10.1093/jb/mvq096.
26. Lin S, Gregory RI. MicroRNA biogenesis pathways in cancer. Nat Rev Cancer. 2015;15:321–33. https://doi.org/10.1038/nrc3932.
27. Blahna MT, Hata A. Regulation of miRNA biogenesis as an integrated component of growth factor signaling. Curr Opin Cell Biol. 2013;25:233–40. https://doi.org/10.1016/j.ceb.2012.12.005.
28. Gurtan AM, Sharp PA. The role of miRNAs in regulating gene expression networks. J Mol Biol. 2013;425:3582–600. https://doi.org/10.1016/j.jmb.2013.03.007.
29. Avraham R, Yarden Y. Regulation of signalling by microRNAs. Biochem Soc Trans. 2012;40:26–30. https://doi.org/10.1042/BST20110623.
30. Shi XB, Tepper CG, deVere White RW. Cancerous miRNAs and their regulation. Cell Cycle. 2008;7:1529–38.
31. Pichler M, Calin GA. MicroRNAs in cancer: from developmental genes in worms to their clinical application in patients. Br J Cancer. 2015;113:569–73. https://doi.org/10.1038/bjc.2015.253.
32. Calin GA, Dumitru CD, Shimizu M, et al. Frequent deletions and down-regulation of micro-RNA genes mir15 and mir16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A. 2002;99:15524–9.
33. Di Leva G, Croce CM. Roles of small RNAs in tumor formation. Trends Mol Med. 2010;16:257–67.
34. Mendell JT, Olson EN. MicroRNAs in stress signaling and human disease. Cell. 2012;148:1172–87.
35. He L, Thomson JM, Hemann MT, et al. A microRNA polycistron as a potential human oncogene. Nature. 2005;435:828–33.
36. Kim HH, Kuwano Y, Srikantan S, et al. HuR recruits let-7/RISC to repress c-Myc expression. Genes Dev. 2009;23:1743–8. https://doi.org/10.1101/gad.1812509.
37. Johnson SM, Grosshans H, Shingara J, et al. RAS is regulated by the let-7 microRNA family. Cell. 2005;120:635–47.
38. Kumar MS, Erkeland SJ, Pester RE, et al. Suppression of non-small cell lung tumor development by the let-7 microRNA family. Proc Natl Acad Sci U S A. 2008;105:3903–8. https://doi.org/10.1073/pnas.0712321105.

39. Svoronos AA, Engelman DM, Slack FJ. OncomiR or tumor suppressor? The duplicity of MicroRNAs in cancer. Cancer Res. 2016;76:3666–70. https://doi.org/10.1158/0008-5472.CAN-16-0359.
40. Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. Nature. 2005;435:834–8.
41. arube Y, Tanaka H, Osada H, et al. Reduced expression of Dicer associated with poor prognosis in lung cancer patients. Cancer Sci. 2005;96:111–5.
42. Lin RJ, Lin YC, Chen J, et al. MicroRNA signature and expression of Dicer and Drosha can predict prognosis and delineate risk groups in neuroblastoma. Cancer Res. 2010;70:7841–50. https://doi.org/10.1158/0008-5472.CAN-10-0970.
43. Merritt WM, Lin YG, Han LY, et al. Dicer, Drosha, and outcomes in patients with ovarian cancer. N Engl J Med. 2008;359:2641–50. https://doi.org/10.1056/NEJMoa0803785.
44. Kumar MS, Lu J, Mercer KL, et al. Impaired microRNA processing enhances cellular transformation and tumorigenesis. Nat Genet. 2007;39:673–7.
45. Hill DA, Ivanovich J, Priest JR, et al. DICER1 mutations in familial pleuropulmonary blastoma. Science. 2009;325:965. https://doi.org/10.1126/science.1174334.
46. Foulkes WD, Priest JR, Duchaine TF. DICER1: mutations, microRNAs and mechanisms. Nat Rev Cancer. 2014;14:662–72. https://doi.org/10.1038/nrc3802.
47. Rakheja D, Chen KS, Liu Y, et al. Somatic mutations in DROSHA and DICER1 impair microRNA biogenesis through distinct mechanisms in Wilms tumours. Nat Commun. 2014;2:4802. https://doi.org/10.1038/ncomms5802.
48. Torrezan GT, Ferreira EN, Nakahata AM, et al. Recurrent somatic mutation in DROSHA induces microRNA profile changes in Wilms tumour. Nat Commun. 2014;5:4039. https://doi.org/10.1038/ncomms5039.
49. Wegert J, Ishaque N, Vardapour R, et al. Mutations in the SIX1/2 pathway and the DROSHA/DGCR8 miRNA microprocessor complex underlie high-risk blastemal type Wilms tumors. Cancer Cell. 2015;27:298–311. https://doi.org/10.1016/j.ccell.2015.01.002.
50. Walz AL, Ooms A, Gadd S, et al. Recurrent DGCR8, DROSHA, and SIX homeodomain mutations in favorable histology Wilms tumors. Cancer Cell. 2015;27:286–97. https://doi.org/10.1016/j.ccell.2015.01.003.
51. Webster RJ, Giles KM, Price KJ. Regulation of epidermal growth factor receptor signaling in human cancer cells by microRNA-7. J Biol Chem. 2009;284:5731–41. https://doi.org/10.1074/jbc.M804280200.
52. Kefas B, Godlewski J, Comeau L, et al. MicroRNA-7 inhibits the epidermal growth factor receptor and the akt pathway and is down-regulated in glioblastoma. Cancer Res. 2008;68:3566–72. https://doi.org/10.1158/0008-5472.CAN-07-6639.
53. Weiss GJ, Bemis LT, Nakajima E, et al. EGFR regulation by microRNA in lung cancer: correlation with clinical response and survival to gefitinib and EGFR expression in cell lines. Ann Oncol. 2008;19:1053–9. https://doi.org/10.1093/annonc/mdn006.
54. Chiyomaru T, Seki N, Inoguchi S, et al. Dual regulation of receptor tyrosine kinase genes EGFR and c-met by the tumor-suppressive microRNA-23b/27b cluster in bladder cancer. Int J Oncol. 2015;46:487–96. https://doi.org/10.3892/ijo.2014.2752.
55. Wang LK, Hsiao TH, Hong TM, et al. MicroRNA-133a suppresses multiple oncogenic membrane receptors and cell invasion in non-small cell lung carcinoma. PLoS One. 2014;9:e96765. https://doi.org/10.1371/journal.pone.0096765.
56. Cui W, Zhang S, Shan C, et al. MicroRNA-133a regulates the cell cycle and proliferation of breast cancer cells by targeting epidermal growth factor receptor through the EGFR/AKT signaling pathway. FEBS J. 2013;280:3962–74. https://doi.org/10.1111/febs.12398.
57. Liu L, Shao X, Gao W, et al. MicroRNA-133b inhibits the growth of non-small-cell lung cancer by targeting the epidermal growth factor receptor. FEBS J. 2012;279:3800–12. https://doi.org/10.1111/j.1742-4658.2012.08741.x.

58. Kumaraswamy E, Wendt KL, Augustine LA, et al. BRCA1 regulation of epidermal growth factor receptor (EGFR) expression in human breast cancer cells involves microRNA-146a and is critical for its tumor suppressor function. Oncogene. 2014;34:4333–46. https://doi.org/10.1038/onc.2014.363.
59. Katakowski M, Zheng X, Jiang F, et al. Mir-146b-5p suppresses EGFR expression and reduces in vitro migration and invasion of glioma. Cancer Invest. 2010;28:1024–30. https://doi.org/10.3109/07357907.2010.512596.
60. Rao SA, Arimappamagan A, Pandey P, et al. Mir-219-5p inhibits receptor tyrosine kinase pathway by targeting EGFR in glioblastoma. PLoS One. 2013;8:e63164. https://doi.org/10.1371/journal.pone.0063164.
61. Wang L, Yao J, Shi X, et al. MicroRNA-302b suppresses cell proliferation by targeting EGFR in human hepatocellular carcinoma SMMC-7721 cells. BMC Cancer. 2013;13:448. https://doi.org/10.1186/1471-2407-13-448.
62. Zhang Y, Schiff D, Park D, et al. MicroRNA-608 and microRNA-34a regulate chordoma malignancy by targeting EGFR, Bcl-xL and MET. PLoS One. 2014;9:e91546. https://doi.org/10.1371/journal.pone.0091546.
63. Uhlmann S, Mannsperger H, Zhang JD, et al. Global microRNA level regulation of EGFR-driven cell-cycle protein network in breast cancer. Mol Syst Biol. 2012;8:570. https://doi.org/10.1038/msb.2011.100.
64. Pagliuca A, Valvo C, Fabrizi E, et al. Analysis of the combined action of mir-143 and mir-145 on oncogenic pathways in colorectal cancer cells reveals a coordinate program of gene repression. Oncogene. 2013;32:4806–13. https://doi.org/10.1038/onc.2012.495.
65. Pekow JR, Dougherty U, Mustafi R, et al. Mir-143 and mir-145 are downregulated in ulcerative colitis: putative regulators of inflammation and protooncogenes. Inflamm Bowel Dis. 2012;18:94–100. https://doi.org/10.1002/ibd.21742.
66. Xu B, Niu X, Zhang X, et al. Mir-143 decreases prostate cancer cells proliferation and migration and enhances their sensitivity to docetaxel through suppression of kras. Mol Cell Biochem. 2011;350:207–13. https://doi.org/10.1007/s11010-010-0700-6.
67. Wu X, Bhayani MK, Dodge CT, et al. Coordinated targeting of the EGFR signaling axis by microrna-27a*. Oncotarget. 2013;4:1388–98.
68. Leivonen SK, Sahlberg KK, Makela R, et al. High-throughput screens identify microRNAs essential for HER2 positive breast cancer cell growth. Mol Oncol. 2014;8:93–104. https://doi.org/10.1016/j.molonc.2013.10.001.
69. Giles KM, Barker A, Zhang PM, et al. MicroRNA regulation of growth factor receptor signaling in human cancer cells. Methods Mol Biol. 2011;676:147–63.
70. Epis MR, Giles KM, Barker A, et al. Mir-331-3p regulates ERBB2 expression and androgen receptor signaling in prostate cancer. J Biol Chem. 2009;284:24696–704. https://doi.org/10.1074/jbc.M109.030098.
71. Bischoff A, Bayerlova M, Strotbek M, et al. A global microRNA screen identifies regulators of the ERBB receptor signaling network. Cell Commun Signal. 2015;13:5. https://doi.org/10.1186/s12964-015-0084-z.
72. Scott GK, Goga A, Bhaumik D, et al. Coordinate suppression of ERBB2 and ERBB3 by enforced expression of microRNA mir-125a or mir-125b. J Biol Chem. 2007;282:1479–86. https://doi.org/10.1074/jbc.M609383200.
73. Liang H, Liu M, Yan X, et al. Mir-193a-3p functions as a tumor suppressor in lung cancer by down-regulating ERBB4. J Biol Chem. 2015;290:926–40. https://doi.org/10.1074/jbc.M114.621409.
74. Yu T, Li J, Yan M, et al. MicroRNA-193a-3p and -5p suppress the metastasis of human non-small-cell lung cancer by downregulating the ERBB4/PIK3R3/mTOR/S6K2 signaling pathway. Oncogene. 2015;34:413–23. https://doi.org/10.1038/onc.2013.574.
75. Zhang M, Yang Q, Zhang L, et al. Mir-302b is a potential molecular marker of esophageal squamous cell carcinoma and functions as a tumor suppressor by targeting ERBB4. J Exp Clin Cancer Res. 2014;33:10. https://doi.org/10.1186/1756-9966-33-10.

76. Guo L, Zhang Y, Zhang L, et al. MicroRNAs, TGF-β signaling, and the inflammatory microenvironment in cancer. Tumour Biol. 2016;37:115–25. https://doi.org/10.1007/s13277-015-4374-2.
77. Butz H, Rácz K, Hunyady L, et al. Crosstalk between TGF-β signaling and the microRNA machinery. Trends Pharmacol Sci. 2012;33:382–93. https://doi.org/10.1016/j.tips.2012.04.003.
78. Sivadas VP, Kannan S. The microRNA networks of TGF-β signaling in cancer. Tumour Biol. 2014;35:2857–69. https://doi.org/10.1007/s13277-013-1481-9.
79. Martin J, Jenkins RH, Bennagi R, et al. Post-transcriptional regulation of transforming growth factor beta-1 by microRNA-744. PLoS One. 2011;6:e25044. https://doi.org/10.1371/journal.pone.0025044.
80. Dogar AM, Towbin H, Hall J. Suppression of latent transforming growth factor (TGF)-β1 restores growth inhibitory TGF-β signaling through microRNAs. J Biol Chem. 2011;286:16447–58. https://doi.org/10.1074/jbc.M110.208652.
81. Dogar AM, Semplicio G, Guennewig B, et al. Multiple microRNAs derived from chemically synthesized precursors regulate thrombospondin 1 expression. Nucleic Acid Ther. 2014;24:149–59. https://doi.org/10.1089/nat.2013.0467.
82. Braun J, Hoang-Vu C, Dralle H, et al. Downregulation of microRNAs directs the EMT and invasive potential of anaplastic thyroid carcinomas. Oncogene. 2010;29:4237–44. https://doi.org/10.1038/onc.2010.169.
83. Masri S, Liu Z, Phung S, et al. The role of microRNA-128a in regulating TGF-beta signaling in letrozole-resistant breast cancer cells. Breast Cancer Res Treat. 2010;124:89–99. https://doi.org/10.1007/s10549-009-0716-3.
84. Li S, Zhang H, Wang X. Direct targeting of HGF by miR-16 regulates proliferation and migration in gastric cancer. Tumour Biol. 2016;37:15175–83.
85. Chen QY, Jiao DM, YQ W. MiR-206 inhibits HGF-induced epithelial-mesenchymal transition and angiogenesis in non-small cell lung cancer via c-met/PI3k/Akt/mTOR pathway. Oncotarget. 2016;7:18247–61. 10.18632/oncotarget.7570.
86. Yang X, Zhang XF, Lu X. MicroRNA-26a suppresses angiogenesis in human hepatocellular carcinoma by targeting hepatocyte growth factor-cMet pathway. Hepatology. 2014;59:1874–85. https://doi.org/10.1002/hep.26941.
87. Tan S, Li R, Ding K. miR-198 inhibits migration and invasion of hepatocellular carcinoma cells by targeting the HGF/c-MET pathway. FEBS Lett. 2011;585:2229–34. https://doi.org/10.1016/j.febslet.2011.05.042.
88. Rokavee M, Li H, Jiang L, et al. The p53/miR-34 axis in development and disease. J Mol Cell Biol. 2014;6:214–30.
89. Brighenti M. MicroRNA and MET in lung cancer. Ann Transl Med. 2014;3:68. https://doi.org/10.3978/j.issn.2305-5839.2015.01.26.
90. Jung HJ, Suh Y. Regulation of IGF-1 signaling by microRNAs. Front Genet. 2015;5:472. https://doi.org/10.3389/fgene.2014.00472.
91. Correia AC, Moonen JR, Brinker MG. FGF2 inhibits endothelial-mesenchymal transition through microRNA-20a-mediated repression of canonical TGF-β signaling. J Cell Sci. 2016;129:569–79. https://doi.org/10.1242/jcs.176248.
92. Xiao J, Bei Y, Liu J. miR-212 downregulation contributes to the protective effect of exercise against non-alcoholic fatty liver via targeting FGF-21. J Cell Mol Med. 2016;20:204–16. https://doi.org/10.1111/jcmm.12733.
93. Chen XY, Li GM, Dong Q. MiR-577 inhibits pancreatic β-cell function and survival by targeting fibroblast growth factor 21 (FGF-21) in pediatric diabetes. Genet Mol Res. 2015;14:15462–70. https://doi.org/10.4238/2015.November.30.24.
94. Wang L, Ma L, Fan H. MicroRNA-9 regulates cardiac fibrosis by targeting PDGFR-β in rats. J Physiol Biochem. 2016;72:213–23. https://doi.org/10.1007/s13105-016-0471-y.
95. Wang W, Zhang E, Lin C. MicroRNAs in tumor angiogenesis. Life Sci. 2015;136:28–35.

96. Landskroner-Eiger S, Moneke I, Sessa WC. miRNAs as modulators of angiogenesis. Cold Spring Harb Perspect Med. 2013;3:a006643.
97. Ohnishi T, Daikuhara Y. Hepatocyte growth factor/scatter factor in development, inflammation and carcinogenesis: its expression and role in oral tissues. Arch Oral Biol. 2003;48:797–804.
98. Uchida D, Kawamata H, Omotehara F, et al. Role of HGF/c-MET system in invasion and metastasis of oral squamous cell carcinoma cells in vitro and its clinical significance. Int J Cancer. 2001;93:489–96.
99. Knowles LM, Stabile LP, Egloff AM, et al. HGF and c-met participate in paracrine tumorigenic pathways in head and neck squamous cell cancer. Clin Cancer Res. 2009;15:3740–50.
100. Matsumoto K, Nakamura T. Hepatocyte growth factor and the met system as a mediator of tumor-stromal interactions. Int J Cancer. 2006;119:477–83.
101. Ding W, You H, Dang H, et al. Epithelial-to-mesenchymal transition of murine liver tumor cells promotes invasion. Hepatology. 2010;52:945–53.
102. Morello S, Olivero M, Aimetti M, et al. MET receptor is overexpressed but not mutated in oral squamous cell carcinoma. J Cell Physiol. 2001;189:285–90.
103. Ren Y, Cao B, Law S et al (2005) Hepatocyte growth factor promotes cancer cell migration and angiogenic factors expression: a prognostic marker of human esophageal squamous cell carcinomas. Clin Cancer Res11:6190–6197.
104. Dong G, Chen Z, Li ZY, et al. Hepatocyte growth factor/scatter factor-induced activation of MEK and PI3K signal pathways contributes to expression of proangiogenic cytokines interleukin-8 and vascular endothelial growth factor in head and neck squamous cell carcinoma. Cancer Res. 2001;61:5911–8.
105. Hanzawa M, Shindoh M, Higashino F, et al. Hepatocyte growth factor upregulates E1AF that induces oral squamous cell carcinoma cell invasion by activating matrix metalloproteinase genes. Carcinogenesis. 2000;21:1079–85.
106. Susuki D, Kimura S, Naganuma S, et al. Regulation of microRNA expression by hepatocyte growth factor in head and neck squamous cell carcinoma. Cancer Sci. 2011;102:2164–71. https://doi.org/10.1111/j.1349-7006.2011.02096.x.
107. Gregory PA, Bert AG, Paterson EL, et al. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat Cell Biol. 2008;10:593–601.
108. Bracken CP, Gregory PA, Kolesnikoff N, et al. A double-negative feedback loop between ZEB1-SIP1 and the microRNA-200 family regulates epithelial-mesenchymal transition. Cancer Res. 2008;68:7846–54.
109. Wang Y, Rathinam R, Walch A, et al. ST14(suppression of tumorigenicity 14) gene is a target for miR-27b, and the inhibitory effect of ST14 on cell growth is independent of miR-27b regulation. J Biol Chem. 2009;284:23094–106.
110. Santin AD, Cane S, Bellone S, et al. The novel serine proteas tumor-associated differentially expressed gene-15 (matriptase/MT-SP1) is highly overexpressed in cervical carcinoma. Cancer. 2003;98:1898–904.
111. Lee SL, Huang PY, Roller P, et al. Matriptase/epithin participates in mammary epithelial cell growth and morphogenesis through HGF activation. Mech Dev. 2010;127:82–95.
112. Ding KF, Sun LF, Ge WT, et al. Effect of SNC19/ST14 gene expression on invasion of colorectal cancer cells. World J Gastroenterol. 2005;11:5651–4.
113. List K, Szabo R, Molinolo A, et al. Delineation of matriptase protein expression by enzymatic gene trapping suggests diverging roles in barrier function, hair formation, and squamous cell carcinogenesis. Am J Pathol. 2006;168:1513–25.
114. Zhang B, Pan X, Cobb GP, et al. MicroRNAs as oncogenes and tumor suppressors. Dev Biol. 2006;289:3–16.
115. Esquela-Kerscher A, Slack FJ. Oncomirs——microRNAs with a role in cancer. Nat Rev Cancer. 2006;6:259–69.
116. Tamaru Y, Hayashizaki Y. Cancer research with non-coding RNA. Cancer Sci. 2006;97:1285–90.

117. Hoa M, Davis SL, Ames SJ, et al. Amplification of wild-type K-ras promotes growth of head and neck squamous cell carcinoma. Cancer Res. 2002;62:7154–6.
118. Weidhaas JB, Babar I, Nallur SM, et al. MicroRNA as potential agents to alter resistance to cytotoxic anticancer therapy. Cancer Res. 2007;67:11111–6.
119. Cimmino A, Calin GA, Fabbri M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci U S A. 2005;102:13944–9.
120. Gao P, Tchernyshyov I, Chang TC, et al. C-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. Nature. 2009;458:762–5.
121. Mouillet JF, Chu T, Nelson DM, et al. MiR-205 silences MED1 in hypoxic primary human trophoblasts. FASEB J. 2010;24:2030–9.
122. Mertens-Talcott SU, Chintharlapalli S, Li X, et al. The oncogenic microRNA-27a targets genes that regulate specificity protein transcription factors and the G2-M checkpoint in MDA-MB-231 breast cancer cells. Cancer Res. 2007;67:11001–11.
123. Zhang H, Li M, Han Y, et al. Down-regulation of miR-27a might reverse multidrug resistance of esophageal squamous cell carcinoma. Dig Dis Sci. 2010;55:2545–51.
124. Iorio MV, Casalini P, Piovan C, et al. microRNA-205 regulates HER3 in human breast cancer. Cancer Res. 2009;69:2195–200.
125. Childs G, Fazzari M, Kung G, et al. Low-level expression of microRNAs let-7d and miR-205 are prognostic markers of head and neck squamous cell carcinoma. Am J Pathol. 2009;174:736–45.
126. Zidar N, Boštjančič E, Gale N, et al. Down-regulation of microRNAs of the miR-200 family and miR-205, and an altered expression of classic and desmosomal cadherins in spindle cell carcinoma of the head and neck-hallmark of epithelial-mesenchymal transition. Hum Pathol. 2011;42:482–8. https://doi.org/10.1016/j.humpath.2010.07.020.
127. Avraham R, Sas-Chen A, Manor O, et al. EGF decreases the abundance of microRNAs that restrain oncogenic transcription factors. Sci Signal. 2010;3:ra43. https://doi.org/10.1126/scisignal.2000876.
128. Kedmi M, Ben-Chetrit N, Korner C et al (2015) EGF induces microRNAs that target suppressors of cell migration: Mir-15b targets MTSS1 in breast cancer. Sci Signal 8:ra29. doi: https://doi.org/10.1126/scisignal.2005866.
129. Tarcic G, Avraham R, Pines G, et al. EGR1 and the ERK-ERF axis drive mammary cell migration in response to EGF. FASEB J. 2012;26:1582–92. https://doi.org/10.1096/fj.11-194654.
130. Katz M, Amit I, Citri A, et al. A reciprocal tensin-3-cten switch mediates EGF-driven mammary cell migration. Nat Cell Biol. 2007;9:961–9. https://doi.org/10.1038/ncb1622.
131. Llorens F, Hummel M, Pantano L, et al. Microarray and deep sequencing cross-platform analysis of the mirrnome and isomir variation in response to epidermal growth factor. BMC Genomics. 2013;14:371. https://doi.org/10.1186/1471-2164-14-371.
132. WC L, Kao SY, Yang CC, et al. EGF up-regulates mir-31 through the C/EBPbeta signal cascade in oral carcinoma. PLoS One. 2014;9:e108049. https://doi.org/10.1371/journal.pone.0108.
133. Ben-Chetrit N, Chetrit D, Russell R, et al. Synaptojanin 2 is a druggable mediator of metastasis and the gene is overexpressed and amplified in breast cancer. Sci Signal. 2015;8:ra7. https://doi.org/10.1126/scisignal.2005537.
134. Alanazi I, Hoffmann P, Adelson DL. MicroRNAs are part of the regulatory network that controls EGF induced apoptosis, including elements of the JAK/STAT pathway, in a431 cells. PLoS One. 2015;10:e0120337. https://doi.org/10.1371/journal.pone.0120337.
135. Gomez GG, Volinia S, Croce CM, et al. Suppression of microRNA-9 by mutant EGFR signaling upregulates FOXp1 to enhance glioblastoma tumorigenicity. Cancer Res. 2014;74:1429–39. https://doi.org/10.1158/0008-5472.CAN-13-2117.
136. Kao CJ, Martiniez A, Shi XB, et al. Mir-30 as a tumor suppressor connects EGF/src signal to ERG and EMT. Oncogene. 2014;33:2495–503. https://doi.org/10.1038/onc.2013.200.
137. Jazbutyte V, Thum T. MicroRNA-21: From cancer to cardiovascular disease. Curr Drug Targets. 2010;11:926–35.

138. Asangani IA, Rasheed SAK, Nikolova DA, et al. MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor PDCD4 and stimulates invasion, intravasation and metastasis in colorectal cancer. Oncogene. 2007;27:2128–36.
139. Zhu S, Si M-L, Wu H, et al. MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1). J Biol Chem. 2007;282:14328–36.
140. Meng F, Henson R, etal W–JH. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. Gastroenterology. 2007;133:647–58.
141. Pan X, Wang Z-X, Wang R. MicroRNA-21: a novel therapeutic target in human cancer. Cancer Biol Ther. 2010;10:1224–32.
142. Sampson VB, Rong NH, Han J, et al. MicroRNA let-7a down-regulates myc and reverts myc-induced growth in Burkitt lymphoma cells. Cancer Res. 2007;67:9762–70.
143. Mayr C, Hemann MT, Bartel DP. Disrupting the pairing between let-7 and HMGA2 enhances oncogenic transformation. Science. 2007;315:1576–9.
144. Paroo Z, Ye X, Chen S, et al. Phosphorylation of the human microRNA-generating complex mediates MAPK/ERK signaling. Cell. 2009;139:112–21.
145. Blahna MT, Hata A. Smad-mediated regulation of microRNA biosynthesis. FEBS Lett. 2012;586:1906–12. https://doi.org/10.1016/j.febslet.2012.01.041.
146. Hata A, Davis BN. Control of microRNA biogenesis by tgfbeta signaling pathway-a novel role of smads in the nucleus. Cytokine Growth Factor Rev. 2009;20:517–21. https://doi.org/10.1016/j.cytogfr.2009.10.004.
147. Davis BN, Hilyard AC, etal LG. Smad proteins control DROSHA-mediated microRNA maturation. Nature. 2008;454:56–61. https://doi.org/10.1038/nature07086.
148. Heldin CH, Moustakas A. Role of smads in TGF-beta signaling. Cell Tissue Res. 2011;347:21–36. https://doi.org/10.1007/s00441-011-1190-x.
149. Davis BN, Hilyard AC, Nguyen PH, et al. Smad proteins bind a conserved RNA sequence to promote microRNA maturation by drosha. Mol Cell. 2010;39:373–84. https://doi.org/10.1016/j.molcel.2010.07.011.
150. Wang W, Li J, etal ZW. MicroRNA-21 and the clinical outcomes of various carcinomas: a systematic review and meta-analysis. BMC Cancer. 2014;14:819. https://doi.org/10.1186/1471-2407-14-819.
151. Yu Y, Wang Y, Ren X, et al. Context-dependent bidirectional regulation of the muts homolog 2 by transforming growth factor beta contributes to chemoresistance in breast cancer cells. Mol Cancer Res. 2010;8:1633–42. https://doi.org/10.1158/1541-7786.MCR-10-0362.
152. Wang B, Hsu SH, Majumder S, et al. TGF-beta-mediated upregulation of hepatic mir-181b promotes hepatocarcinogenesis by targeting TIMP3. Oncogene. 2010;29:1787–97. https://doi.org/10.1038/onc.2009.468.
153. Liu Y, Lai L, Chen Q, et al. MicroRNA-494 is required for the accumulation and functions of tumor-expanded myeloid-derived suppressor cells via targeting of PTEN. J Immunol. 2012;188:5500–10. https://doi.org/10.4049/jimmunol.1103505.
154. Ma L, Reinhardt F, Pan E, et al. Therapeutic silencing of mir-10b inhibits metastasis in a mouse mammary tumor model. Nat Biotechnol. 2010;28:341–7. https://doi.org/10.1038/nbt.1618.
155. Ma L. Role of mir-10b in breast cancer metastasis. Breast Cancer Res. 2010;12:210. https://doi.org/10.1186/bcr2720.
156. Cheng CM, Shiah SG, Huang CC, et al. Up-regulation of miR-455-5p by the TGF-β-SMAD signalling axis promotes the proliferation of oral squamous cancer cells by targeting UBE2B. J Pathol. 2016;240:38–49. https://doi.org/10.1002/path.4752.
157. Yang P, Li Q-J, Feng Y, et al. TGF-β-mir-34a-ccl22 signaling-induced Treg cell recruitment promotes venous metastases of HBV-positive hepatocellular carcinoma. Cancer Cell. 2012;22:291–303. https://doi.org/10.1016/j.ccr.2012.07.023.
158. Xu M, Gu M, Zhang K, et al. Mir-203 inhibition of renal cancer cell proliferation, migration and invasion by targeting of FGF2. Diagn Pathol. 2015;10:24. https://doi.org/10.1186/s13000-015-0255-7.

159. Ding X, Park SI, McCauley LK, et al. Signaling between transforming growth factor beta (TGF-β) and transcription factor SNAL2 represses expression of microRNA mir-203 to promote epithelial-mesenchymal transition and tumor metastasis. J Biol Chem. 2013;288:10241–53. https://doi.org/10.1074/jbc.M112.443655.
160. Fils-Aime N, Dai M, Guo J, et al. MicroRNA-584 and the protein phosphatase and actin regulator 1 (PHACTR1), a new signaling route through which transforming growth factor-beta mediates the migration and actin dynamics of breast cancer cells. J Biol Chem. 2013;288:11807–23. https://doi.org/10.1074/jbc.M112.430934.
161. Sun MM, Li JF, Guo LL, et al. TGFβ1 suppression of microRNA-450b-5p expression: a novel mechanism for blocking myogenic differentiation of rhabdomyosarcoma. Oncogene. 2014;33:2075–86. https://doi.org/10.1038/onc.2013.165.
162. Chen PY, Qin L, Barnes C, et al. FGF regulates TGF-β signaling and endothelial-to-mesenchymal transition via control of let-7 miRNA expression. Cell Rep. 2012;2:1684–96. https://doi.org/10.1016/j.celrep.2012.10.021.
163. Lee JY, Yun M, Paik JS, et al. PDGF-BB enhances the proliferation of cells in human orbital fibroblasts by suppressing PDCD4 expression via up-regulation of microRNA-21. Invest Ophthalmol Vis Sci. 2016;57:908–13. https://doi.org/10.1167/iovs.15-18157.
164. Kim S, Kang H. miR-15b induced by platelet-derived growth factor signaling is required for vascular smooth muscle cell proliferation. BMB Rep. 2013;46:550–4.
165. Cho WC, Chow AS, Au .S (2011) Mir-145 inhibits cell proliferation of human lung adenocarcinoma by targeting EGFR and NUDT1. RNA Biol 8:125–131. doi: https://doi.org/10.4161/rna.8.1.14259.
166. Zhu H, Dougherty U, Robinson V, et al. EGFR signals downregulate tumor suppressors mir-143 and mir-145 in western diet-promoted murine colon cancer: role of G1 regulators. Mol Cancer Res. 2011;9:960–75. https://doi.org/10.1158/1541-7786.MCR-10-0531.
167. Guo YH, Zhang C, Shi J, et al. Abnormal activation of the EGFR signaling pathway mediates the downregulation of mir145 through the ERK1/2 in non-small cell lung cancer. Oncol Rep. 2014;31:1940–6.
168. Zhang KL, Han L, Chen LY, et al. Blockage of a mir-21/EGFR regulatory feedback loop augments anti-EGFR therapy in glioblastomas. Cancer Lett. 2014;342:139–49. https://doi.org/10.1016/j.canlet.2013.08.043.
169. Seike M, Goto A, Okano T, et al. Mir-21 is an EGFR-regulated anti-apoptotic factor in lung cancer in never-smokers. Proc Natl Acad Sci U S A. 2009;106:12085–90. https://doi.org/10.1073/pnas.0905234106.
170. Perou CM, Sorlie T, Eisen MB, et al. Molecular portraits of human breast tumours. Nature. 2000;406:747–52. https://doi.org/10.1038/35021093.
171. Volinia S, Calin GA, Liu CG, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A. 2006;103:2257–61. https://doi.org/10.1073/pnas.0510565103.
172. Mitchell PS, Parkin RK, Kroh EM, et al. Circulating micrornas as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A. 2008;105:10513–8. https://doi.org/10.1073/pnas.0804549105.
173. Chen X, Ba Y, Ma L, et al. Characterization of micrornas in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res. 2008;18:997–1006. https://doi.org/10.1038/cr.2008.282.
174. Takamizawa J, Konishi H, Yanagisawa K, et al. Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer Res. 2004;64:3753–6. https://doi.org/10.1158/0008-5472.CAN-04-0637.
175. Nelson PT, Baldwin DA, Scearce LM, et al. Microarray-based, high-throughput gene expression profiling of micrornas. Nat Methods. 2004;1:155–61. https://doi.org/10.1038/nmeth717.
176. Lagos-Quintana M, Rauhut R, Yalcin A, et al. Identification of tissue-specific microRNAs from mouse. Curr Biol. 2002;12:735–9. https://doi.org/10.1016/S0960-9822(02)00809-6.

177. Rosenfeld N, Aharonov R, Meiri E, et al. MicroRNAs accurately identify cancer tissue origin. Nat Biotechnol. 2008;26:462–9. https://doi.org/10.1038/nbt1392.
178. Dvinge H, Git A, Graf S, et al. The shaping and functional consequences of the microRNA landscape in breast cancer. Nature. 2013;497:378–82. https://doi.org/10.1038/nature12108.
179. Blenkiron C, Goldstein LD, Thorne NP, et al. MicroRNA expression profiling of human breast cancer identifies new markers of tumor subtype. Genome Biol. 2007;8:R214. https://doi.org/10.1186/gb-2007-8-10-r214.
180. Andorfer CA, Necela BM, Thompson EA, et al. MicroRNA signatures: clinical biomarkers for the diagnosis and treatment of breast cancer. Trends Mol Med. 2011;17:313–9. https://doi.org/10.1016/j.molmed.2011.01.006.
181. Enerly E, Steinfeld I, Kleivi K, et al. MiRNA-mRNA integrated analysis reveals roles for miRNAs in primary breast tumors. PLoS One. 2011;6:e16915. https://doi.org/10.1371/journal.pone.0016915.
182. Yanaihara N, Caplen N, Bowman E, et al. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell. 2006;9:189–98. https://doi.org/10.1016/j.ccr.2006.01.025.
183. Hu Z, Chen X, Zhao Y, et al. Serum microRNA signatures identified in a genome-wide serum microRNA expression profiling predict survival of non-small-cell lung cancer. J Clin Oncol. 2010;28:1721–6. https://doi.org/10.1200/JCO.2009.24.9342.
184. Cho WC. MicroRNAs: potential biomarkers for cancer diagnosis, prognosis and targets for therapy. Int J Biochem Cell Biol. 2010;42:1273–81. https://doi.org/10.1016/j.biocel.2009.12.014.
185. Meiri E, Mueller WC, Rosenwald S, et al. A second-generation microRNA-based assay for diagnosing tumor tissue origin. Oncologist. 2012;17:801–12. https://doi.org/10.1634/theoncologist.2011-0466.
186. Nair VS, Maeda LS, Ioannidis JP. Clinical outcome prediction by microRNAs in human cancer: a systematic review. J Natl Cancer Inst. 2012;104:528–40. https://doi.org/10.1093/jnci/djs027.

Chapter 4
Regulation of EMT by TGF-β Signaling in Cancer Cells

Masao Saitoh

Abstract The transforming growth factor (TGF)-β pathway mediates a broad spectrum of cellular processes and is involved in several diseases, including cancer. TGF-β can suppress tumorigenesis by inhibiting cell-cycle progression and stimulating apoptosis in the early stages of cancer, suggesting that it acts as a tumor suppressor during cancer initiation. However, TGF-β can also act as a tumor promoter at later stages of cancer progression. TGF-β plays fundamental roles in cancer cells and various types of cells in the cancer microenvironment, leading to angiogenesis, suppression of antitumor immunity, fibroblast differentiation, extracellular matrix deposition, and induction of the epithelial–mesenchymal transition (EMT). The EMT plays crucial roles in appropriate embryonic development and also functions in adults during wound healing, organ fibrosis, and tumor progression. Many secreted factors are implicated in this process. Among them, TGF-β induces the EMT by propagating intracellular signals and activating transcription factors. This review describes new insights into the molecular mechanisms underlying induction of the EMT by TGF-β in cooperation with signals from growth factors and oncogenic signals such as Ras and also discusses the signals that induce the EMT through transcriptional and posttranscriptional regulation.

Keywords TGF-β • Smad • Ras • Snail

Abbreviations

EMT	Epithelial–mesenchymal transition
TGF-β	Transforming growth factor-β
SIP1	Smad-interacting protein 1

M. Saitoh
Department of Biological Chemistry, Center for Medical Education and Sciences, Interdisciplinary Graduate School of Medicine, University of Yamanashi, Yamanashi, Japan
e-mail: msaitoh-ind@umin.ac.jp

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N. Shinomiya et al. (eds.), *Regulation of Signal Transduction in Human Cell Research*, Current Human Cell Research and Applications,
https://doi.org/10.1007/978-981-10-7296-3_4

δEF1	δ-crystallin/E2-box factor 1
R-Smad	Receptor-regulated Smad

4.1 Canonical and Noncanonical Signaling of TGF-β

Transforming growth factor (TGF)-β is a prototypic member of a large superfamily of more than 40 secreted cytokines, including TGF-β1, TGF-β2, TGF-β3, bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), activins, and myostatin [1]. These pleiotropic cytokines regulate numerous biological functions, including proliferation, apoptosis, differentiation, and migration, in various types of cells, thereby regulating a broad spectrum of biological processes in embryos as well as adult tissues [2]. TGF-β is initially synthesized as the inactive pro-TGF-β form, which consists of latency-associated peptides (LAPs) and latent TGF-β-binding proteins (LTBPs) assembled together with disulfide bridges between specific cysteine residues. The TGF-β large latent complex is deposited in the extracellular matrix (ECM) of most tissues and in granules of platelets. It is activated by plasmin and matrix metalloproteinases and by certain environmental conditions, such as low pH levels, reactive oxygen species (ROS) induced by irradiation, and mechanical stresses [3, 4]. Activated TGF-β can bind to transmembrane serine/threonine kinase receptors type I (TβR-I) and type II (TβR-II), which are expressed in almost all cell types, and thus transduce downstream signals. In the canonical TGF-β signaling pathway, TGF-β induces formation of a hetero-tetramer receptor complex, consisting of two TβR-IIs and two TβR-Is, in which TβR-I is phosphorylated and activated by the kinase of TGF-β-bound TβR-II [5]. Following receptor activation, Smad2 and/or Smad3, which associate with the TGF-β receptor complex and are phosphorylated by the kinase of TβR-I, form a complex with Smad4 and translocate to the nucleus. There, the Smad complex regulates the transcription of target genes in cooperation with other nuclear cofactors [6].

Receptor-activated Smads (R-Smads, Smad2, and Smad3 in TGF-β signaling) are phosphorylated at multiple sites by upstream kinases. TβR-I in the TGF-β-bound receptor complex phosphorylates the C-terminal serine residues of R-Smads. On the other hand, mitogenic signals cause phosphorylation of R-Smads at specific sites in the proline-rich acidic linker region, which connects between the N-terminal Mad homology (MH1) domain and the C-terminal MH2 domain. Linker phosphorylation leads to retention in the cytoplasm and promotion of ubiquitin-dependent degradation, resulting in negative regulation of TGF-β signaling [7]. In addition, phosphorylation of the linker region of Smad3 is required to transduce signals independent of TGF-β [8, 9]. Recent work showed that linker phosphorylation at threonine 179 of Smad3 is dispensable for regulation of the alternative splicing machinery dependent on the mRNA-binding protein PCBP1, which is independent of Smad4 [10]. Hence, the physiological significance of phosphorylation of the linker region remains controversial and may be dependent on cellular context.

TGF-β can also transduce signals independent of Smads, the so-called noncanonical signaling pathway. Activation of Erk by TGF-β in epithelial and cancer cells

promotes cell migration, whereas TGF-β inactivates the Erk pathway in some epithelial cells during the EMT [11]. In addition, ShcA activation by TGF-β promotes the formation of ShcA/Grb2/Sos complex and in turn activates Ras signals, resulting in apoptosis or cell migration via various MAPK signaling pathways [12]. Further, the PI3K/Akt and mTOR/S6K pathways have been implicated in mediating some cellular functions of TGF-β [13]. TGF-β can rapidly activate the RhoA and Cdc42/Rac1 pathways to promote cell motility and formation of stress fibers, and RhoA inhibitors suppress the induction of the EMT by TGF-β [14]. Together with numerous reports describing noncanonical pathways involving TGF-β, this evidence suggests that such pathways also play critical roles in regulating TGF-β-mediated cellular functions.

4.2 Dual Roles of TGF-β Signaling in Cancer

TGF-β was initially identified as a factor that induces proliferation and transformation of fibroblasts [15]. TGF-β has a growth-inhibitory effect on normal epithelial, endothelial, neuronal, and immune cells. Genome-wide transcriptional profiling studies performed in normal human epithelial cell lines from the mammary gland, skin, and lung have identified a common set of genes that are transcriptionally regulated by TGF-β in order to mediate its cytostatic effects: induction of the expression of the cyclin-dependent kinase inhibitors CDKN2B (encoding p15/INK4B), CDKN1A (encoding p21/Cip/Waf1), and p27/Kip1 and repression of the family of inhibitor of DNA-binding proteins ID1, ID2, and ID3 and repression of growth-inducing transcription factors, such as c-Myc [16]. In some cases, TGF-β also induces apoptosis of epithelial cells via TGF-β-inducible early response gene-1 (TIEG1), the death-associated protein kinase DAPK, the adaptor protein Daxx, GADD45β, Bim, and ARTS [17]. In mouse models, ectopic expression of dominant-negative TβR-II in the epidermis promotes hyperplasia or malignant conversion of epithelial cells, whereas TGF-β overexpression in the epidermis decreases proliferation of keratinocytes and protects mice from tumorigenesis and hyperplasia. In various types of tumors, TGF-β signaling is impaired by mutations in signaling components. In particular, the Smad4 gene in chromosome 18q21 is deleted or mutated in 60% of pancreatic and 30% of colorectal cancers. Based on these observations, TGF-β predominantly acts as an inhibitor of cell proliferation in most normal tissues and is considered to act as a tumor suppressor during cancer initiation [18].

These genetic alterations and insensitivity to TGF-β are not detected in all types of cancers. In cancer microenvironments containing many kinds of cells, fibroblasts and macrophages differentiate into cancer-associated fibroblasts (CAFs) and tumor-associated macrophages (TAMs), respectively, possibly in response to stimulation by "education" signals, such as cytokines or growth factors, secreted from cancer cells [19]. Microenvironments containing CAFs and TAMs are sometimes called as reactive stroma, which promote motility and proliferation of cancer cells by regulating the expression and secretion of components of the ECM, matrix metalloproteinases (MMPs), and cytokines from stromal cells

[20]. CAFs, which are among the most important cell types in the cancer microenvironment, control cancer cell viability by secreting molecules directed toward cancer cells, known as "reeducation" signals [21]. TGF-β, which is frequently overexpressed in cancer tissues, acts as an "education" signal to regulate cell viability and cellular functions of fibroblasts, immune cells, and endothelial cells in the cancer microenvironment. However, in cooperation with other growth factors or cytokines, it serves as a "reeducation" signal to facilitate proliferation and motile properties of cancer cells by helping them evade immune surveillance and by promoting angiogenesis and lymphangiogenesis. In addition, chronic exposure of cancer cells to TGF-β results in repression of Smad expression and loss of TGF-β-mediated growth inhibition, elevated cell motility and invasion, and marked changes in cell morphology, leading to the epithelial−mesenchymal transition (EMT) [22, 23]. Therefore, TGF-β in cancer tissues promotes cancer progression by modulating cancer cells themselves, as well as normal cells present in cancer microenvironments.

4.3 Transcriptional Factors in the EMT

EMT, a phenotypic conversion that facilitates embryonic development and wound healing during physiological processes, was initially described by developmental biologists [24, 25]. EMT is also associated with the progression of pathological conditions including fibrosis and cancer. EMT involves dramatic cellular changes in which epithelial cells loosen attachments to neighboring cells, acquire an elongated morphology, and display increased motility. In addition, the EMT process is also accompanied by dissolution of adherens junction proteins, such as E-cadherin, β-catenin, γ-catenin, and p120 catenin and by disruption of tight junctions such as Zo-1, occludin, and claudins, resulting in dissociation of epithelial cells and loss of apical−basal polarity. Conversely, the EMT induces expression of mesenchymal marker proteins such as N-cadherin, vimentin, and fibronectin, thereby facilitating attachment to the ECM, and acquisition of mesenchymal features such as spindle-shaped morphology and reorganization of actin stress fibers [26]. Cancer cells undergoing EMT exhibit more aggressive phenotypes, including resistance to drugs and stresses, inhibition of senescence and anoikis, and acquisition of immunosuppression and stem cell-like features (Fig. 4.1). Recent work showed that EMT cells contribute significantly to recurrent metastasis formation after chemotherapy in lung and pancreatic cancer cells [27, 28]. These phenotypic changes are regulated by crucial roles of extracellular matrix components and soluble factors, which regulate several transcription factors, known as key EMT regulators. These include the Snai family of zinc-finger transcription factors (Snail, Slug, and Smuc), the δEF1 family of two-handed zinc-finger factors [δ-crystallin/E2-box factor 1 (δEF1)/zinc-finger E-box-binding homeobox (ZEB) 1 and Smad-interacting protein (SIP) 1/ZEB2], and the basic helix-loop-helix factors Twist and E12/E47.

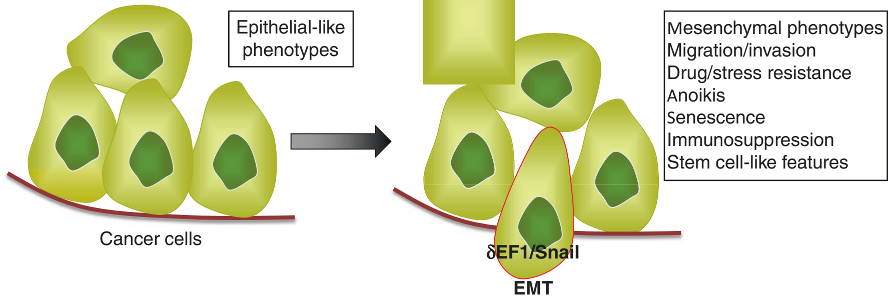

Fig. 4.1 Features of the epithelial–mesenchymal transition (EMT)

4.4 Phosphorylation of Snail and δEF1 During EMT

Posttranslational modifications determine protein fate and localization within the cell. Similar to β-catenin, Snail possesses a typical GSK-3β phosphorylation (Ser-97 and Ser-101) motif within its nuclear export signal and promotes ubiquitin-mediated proteasome degradation by β-TrCP, a process dependent on GSK-3β-mediated phosphorylation [29]. Ser-246 phosphorylation by p21-activated kinase (PAK1) promotes localization of Snail into the nucleus and thereby facilitates its transcription-suppressive effect on E-cadherin [30]. Ser-11 phosphorylation by protein kinase D1 (PKD1) results in its nuclear export and degradation by an E3 ligase, F-box protein 11 (FBXO11) [31]. δEF1 and SIP1 are well-characterized members of the δEF1 family that are involved in the TGF-β-induced EMT in mouse epithelial cells and breast cancer cells [32]. δEF1 and SIP1 were initially identified as molecules that regulate development in mice [33, 34]. δEF1 activates TGF-β signaling by forming a complex with the transcriptional activators p300 and PCAF, whereas SIP1 inhibits signaling by antagonizing R-Smads via direct binding to R-Smads (Postigo, 2003). However, E-cadherin is transcriptionally suppressed by both factors in response to TGF-β. In addition, ERK1/2 phosphorylates δEF1 at Ser-867 and inhibits its transcriptional suppression effect [35]. However, no phosphorylation of SIP1 has been reported to date.

4.5 Upregulation of Snail and δEF1 by TGF-β

Many of these signaling pathways, e.g., Wnt, fibroblast growth factors (FGFs), hepatocyte growth factor (HGF), epidermal growth factor (EGF), TGF-βs, and Notch, promote the EMT during embryonic development and cancer invasion [36, 37]. TGF-β, a key factor in the induction of the EMT, promotes metastasis by upregulating key EMT regulators. The molecular mechanism of TGF-β-induced Snail during EMT in epithelial and cancer cells remains controversial. Snail induction by

TGF-β in mouse epithelial NMuMG cells and pancreatic cancer Panc-1 cells is independent of treatment with cycloheximide, an inhibitor of protein synthesis, suggesting that Snail is a direct target of TGF-β/Smad signaling [32]. However, TGF-β induces Snail in NMuMG cells through the embryonic chromatin factor HMGA2, an immediate-early and direct target of TGF-β/Smad signaling, suggesting that Snail is an indirect target of TGF-β/Smad signaling [38]. Because NMuMG cells show biphasic induction of Snail by TGF-β, Snail may be induced directly and indirectly by TGF-β/Smad signaling at the early and late phase, respectively. TGF-β also induces δEF1 and SIP1 in NMuMG cells and several types of cancer cells through downregulation of Id proteins in a manner dependent on Smads [39, 40]. Because δEF1 and SIP1 function redundantly, silencing of both proteins, but not either one alone, inhibits induction of the EMT by TGF-β [32].

TGF-β also cooperates with oncogenic (constitutively active) K-Ras to induce the EMT, especially expression of Snail. The K-RasG12V mutation has been detected in various types of cancer cells, including Panc-1, lung cancer A549, and prostate cancer PC-3 cells. When K-Ras in these cells is silenced by its specific siRNA, TGF-β fails to induce Snail expression. In HeLa and Madin−Darby canine kidney (MDCK) cells with wild-type K-Ras, TGF-β does not drastically upregulate Snail and can only do so following ectopic expression of RasG12V. When epithelial cells are transformed with oncogenic Ras, they not only become resistant to growth inhibition by TGF-β but also undergo an EMT with invasive and metastatic phenotypes [41, 42]. Oncogenic Ras dramatically enhances Snail expression in collaboration with TGF-β, whereas Ras signaling suppresses TGF-β signaling via linker phosphorylation of R-Smads, inhibits translocation of the R-Smads, and promotes their degradation (Fig. 4.2a) [41]. When four putative phosphorylation sites in the linker region or all serine–proline (S/P) sites in Smad3 are mutated, the resultant mutants can still promote Snail induction (Fig. 4.2b), as in the case of wild-type Ras, suggesting that phosphorylation of S/P sites in Smad3 is indispensable for Snail induction by TGF-β. In a screen of an siRNA library targeting transcription factors, signal transducer and activator of transcription 3 (STAT3) was identified as a mediator that synergizes the TGF-β and Ras signals. Overexpression of STAT3 enhanced Snail induction, whereas protein

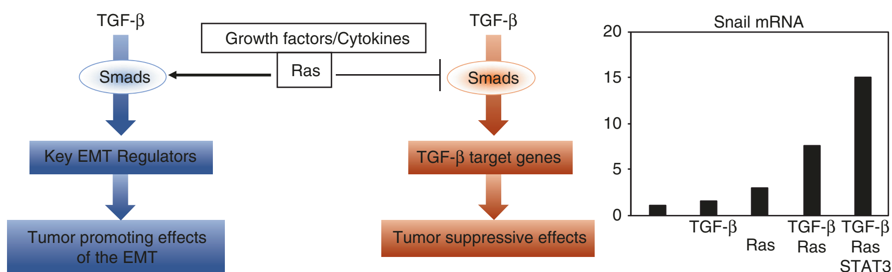

Fig. 4.2 Synergism between TGF-β and other signals for EMT induction. (**a**) RasG12V enhances TGF-β-induced Snail and EMT but suppresses TGF-β-induced Smad7 (a representative target gene of TGF-β) and growth inhibition. (**b**) STAT3 mediates TGF-β-induced Snail expression

inhibitor of activated STAT3 (PIAS3) inhibited the enhancement of Snail promoter activity induced by TGF-β and Ras. Importantly, the putative STAT3-binding elements in the Snail promoter regions were not required for STAT3-mediated Snail induction [43]. The detailed underlying mechanism remains unclear, but STAT3 and PIAS3 are partly involved in Snail induction by TGF-β in cooperation with Ras signals.

4.6 Synergistic Induction of EMT by TGF-β and Other Signals

Cancer invasion/metastasis and EMT are promoted by TGF-β signaling in cooperation with several intracellular signals. Smad complexes induced by TGF-β associate with the transcription coactivators TAZ and YAP, which are regulated by the Hippo pathway [44, 45], and efficiently regulate TGF-β target genes including EMT markers. In addition, Smad3 and Smad4 interact and form a complex with Snail, which also interacts with YAP and TAZ in bone marrow-derived skeletal stem/stromal cell functions [46, 47]. TGF-β activates its type II receptor kinase and then phosphorylates the polarity complex protein Par6 to form a complex with the ubiquitin ligase Smurf1. In turn, Smurf1 ubiquitylates and degrades the small GTPase RhoA, which initiates the EMT through local depolymerization of actin microfilaments and disassembly of tight and adherens junctions [48]. Recent work showed that the atypical protein kinase Cι (PKCι) potentiates the phosphorylation of Par6 by the TβR-II kinase in a complex with the TGF-β receptors [49]. TβR-I induces the EMT, independently of Smad signals, by transducing downstream signals to PI3K/AKT, mTOR, Src tyrosine kinase, p38 MAPK, and focal adhesion kinase (FAK), whose signaling is coupled to activation of the integrin-β1, TRAF6-p300, and the Rho family of small GTPases.

Several growth factors, including FGF2, FGF4, EGF, and HGF, enhance the TGF-β-induced EMT in epithelial and cancer cells [11, 41]. Tumor necrosis factor (TNF)-α also enhances induction of the EMT by TGF-β through NF-kB signals in lung cancer A549 cells [50]. Thus, although many studies reported that the EGF or other growth factors induce the EMT in epithelial cells, as well as cancer cells, we cannot rule out the possibility that the EMT is facilitated by autocrine TGF-β secreted from these cells. Indeed, cancer cells autonomously produce large quantities of TGF-β, and blockade of autocrine TGF-β alters multiple cellular phenomena, including induction of apoptosis by antitumor drugs or serum depletion [51]. Therefore, EMT in cancer cells is regulated by TGF-β secreted from cancer cells themselves in cooperation with other growth factors secreted from cells in the cancer microenvironment, such as CAFs and macrophages. EMT resulting from synergism in cancer cells surrounding the tumor nest may be associated with invasion into the stroma, suggesting that expression of E-cadherin is preferentially repressed at the invasive edge of a metastatic cancer, but not in the central region of the tumor nest. Single cancer cells that invade into tissues can lose E-cadherin expression and

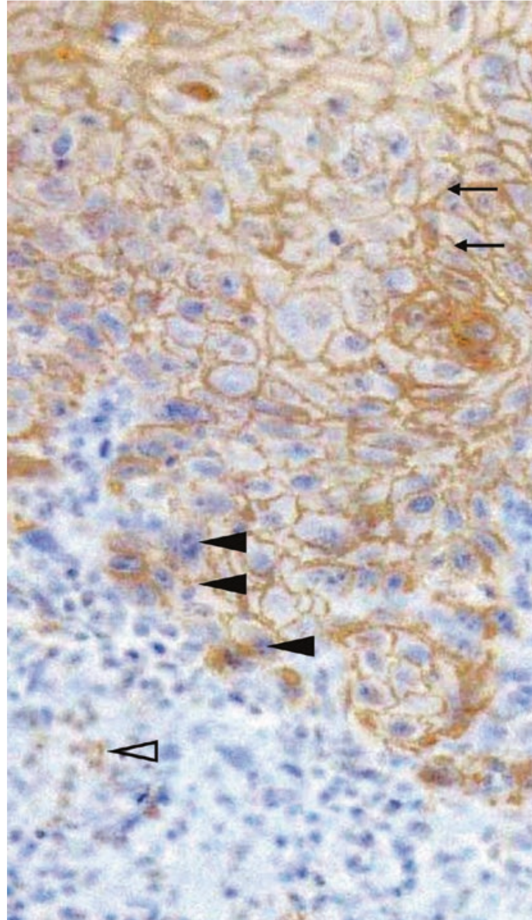

Fig. 4.3 Immunohistological staining with anti-E-cadherin antibody. Arrows indicate the normal appearance of E-cadherin in cancer cells of the tumor nest. Arrowheads in black indicate partial suppression of E-cadherin (partial EMT) in cancer cells at the invasive edge, whereas arrowheads in white indicate almost complete loss of E-cadherin (i.e., complete EMT), probably in the invading cancer cells

increase expression of key EMT regulators, similar to the phenotypes of fibroblasts in stromal tissue [52] (Fig. 4.3).

4.7 Regulation of the EMT at the RNA Level

Several noncoding RNAs, microRNAs (miRNAs), and long noncoding RNAs (lncRNAs) have been shown to play a role in the EMT. In addition, alternative mRNA splicing during the EMT is regulated by the epithelial splicing regulatory proteins ESRP1 and ESRP2. The miRNAs of the miR-200 family target and repress δEF1 and SIP1. TGF-β downregulates the expression of many of the miR-200 family of miRNAs, including miR-200a, miR-200b, miR-200c, miR-141, and miR-429. miR-200 s downregulate expression of the mRNA of TGF-β ligand during the early

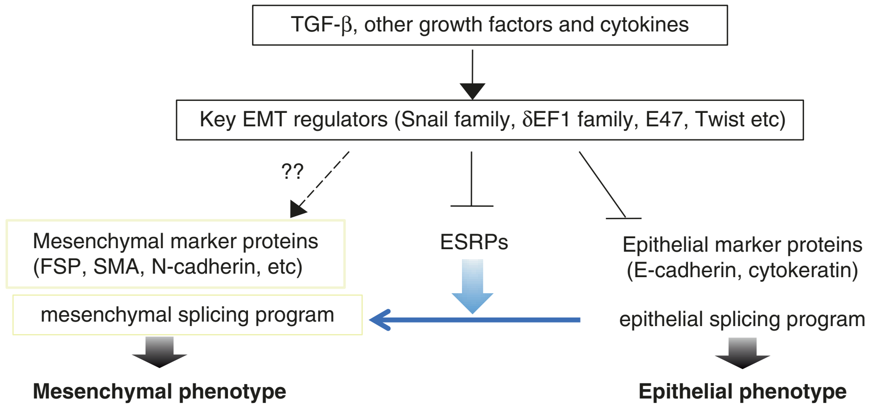

Fig. 4.4 Schematic illustrations of EMT regulated by transcriptional and posttranscriptional mechanisms during induction of the EMT by TGF-β. TGF-β upregulates the key EMT regulators and decreases expression of ESRPs, leading to changes in alternative splicing events. FSP, fibroblast-specific protein; SMA, smooth muscle α-actin

phase of the EMT, generating a regulatory feedback loop [13]. Conversely, TGF-β suppresses miR-200 s via binding of δEF1 and SIP1 to two loci in the miR-200 silencer element, resulting in a double-negative feedback loop (Fig. 4.3) [53]. At the late phase of the EMT, the miR-200 locus becomes hypermethylated [54]. miR-34 and Snail negatively regulate each other [55], as do miR-203 and Slug [56]. miR-99a and miR-99b are induced by TGF-β and then negatively regulate TGF-β signaling by affecting both R-Smad phosphorylation and mTOR [57, 58].

TGF-β signaling regulates the expression of several lncRNAs during the EMT. lncRNA-HIT, lncRNA-ATB, and Malat1 are required for TGF-β-mediated EMT and cancer cell invasion [59–61]. In a recently proposed model known as the "ceRNA theory" [62], miRNAs miR-25 and miR-200 target both the *SIP1* and *PTEN* mRNAs, both of which possess the common miRNA recognition elements (MREs). Attenuation of the *SIP1* mRNA liberates the miRNAs, which then bind to *PTEN* mRNA, resulting in a reduction in PTEN protein levels. Importantly, this process is independent of protein coding. Thus, suppression of *SIP1* mRNA leads to activation of the PI3K/AKT pathway in human cancer cells. Therefore, several types of machinery mediated by ceRNAs might be involved in EMT induction through multiple gene expression networks in cancer cells. However, a ceRNA involved in the TGF-β-induced EMT has not yet been identified.

The alternative splicing machinery is also involved in regulation of the EMT by TGF-β. ESRP1 and ESRP2, also known as RNA recognition motif−containing proteins Rbm35a and Rbm35b, respectively [63], bind directly to hexamers containing repeats of UGG or GGU motifs, which are enriched in alternatively spliced regions. ESRPs induce switching of alternative splicing of FGFRs from the epithelial IIIb isoform to the mesenchymal IIIc isoform. TGF-β downregulates ESRPs during the EMT through direct transcriptional repression by δEF1 and SIP1 and thus sensitizes the cells to FGF-2 and FGF-4, which in turn enhance the EMT in cooperation with

TGF-β [64]. In addition to the FGFRs, ESRPs alter the splicing profiles of CD44, Ste. 20-like kinase, p120 catenin, Rac1, and Mena [a member of the enabled (Ena)/vasodilator-stimulated phosphoprotein (VASP) family of proteins] [65, 66]. The alternative splicing machinery is required for changes in cellular morphology during EMT. Thus, ESRPs play crucial roles in alternative splicing and transcriptional regulation during the EMT in cancer cells.

4.8 Epigenetic Regulation of EMT Regulators

Breast cancers are classified into two subtypes, luminal and basal-like, corresponding to two distinct types of epithelial cells found in the normal mammary gland [67]. The basal-like subtype is associated with aggressive behavior and poor prognosis. Most cell lines with low E-cadherin levels and high δEF1/SIP1 levels are categorized into the basal-like subtype [64]. ESRPs, whose expression patterns are similar to that of E-cadherin, are also expressed at low levels in the basal-like subtype and are repressed by direct binding of δEF1/SIP1 to their promoter regions. Integrin α3 (ITGA3) is highly expressed in the basal-like subtype of breast cancer cells, whereas ESRPs, c-Ret, and RGS16 are poorly expressed in this subtype [52, 68]. Interestingly, in the basal-like subtype, the ERK pathway is constitutively activated and upregulates expression of ITGA3, δEF1, and SIP1. However, the mechanism underlying the high phosphorylation level of ERK1/2 in the basal-like subtype of breast cancer cells has not yet been elucidated. δEF1 interacts with DNA methyltransferase 1 (DNMT1) and some components of the nucleosome remodeling and deacetylase (NuRD) complex, including metastasis-associated proteins (MTAs) and the methyl-CpG-binding domain (MBD) family of proteins [69]. Previous studies reported that Snail also interacts with DNMT1, as well as multiple chromatin-modifying proteins including LSD1 (histone lysine-specific demethylase), PRC2 (polycomb repressive complex 2), and Suv39H1 (histone methyltransferase responsible for the trimethylation of H3K9) [70, 71]. Thus, it seems that δEF1 and Snail regulate both the methylation status and chromatin modification of E-cadherin gene, acting as transcriptional repressors both directly at the transcriptional level and indirectly at the epigenetic level during the EMT associated with cancer progression.

Acknowledgments We would like to thank Dr. K. Sakamoto, Dr. R. Nakamura, Dr. T. Shirakihara, Dr. K. Horiguchi, Dr. K. Miyazono, and the member of Biochemistry Laboratory (University of Yamanashi) for their collaboration. This work was supported by JSPS KAKENHI Grant Number JP15H05018.

Conflict of Interest The authors declare that they have no conflicts of interest.

References

1. Derynck R, Miyazono K. The TGF-β family: Cold Spring Harbor Laboratory Press; 2008.
2. Massague J. TGFbeta in cancer. Cell. 2008;134:215–30.
3. Miyazono K. TGF-beta receptors and signal transduction. Int J Hematol. 1997;65:97–104.
4. Robertson IB, Rifkin DB. Unchaining the beast; insights from structural and evolutionary studies on TGFbeta secretion, sequestration, and activation. Cytokine Growth Factor Rev. 2013;24:355–72.
5. Massague J. TGF-beta signal transduction. Annu Rev Biochem. 1998;67:753–91.
6. Miyazawa K, Shinozaki M, Hara T, Furuya T, Miyazono K. Two major Smad pathways in TGF-beta superfamily signalling. Genes Cells. 2002;7:1191–204.
7. Massague J. Integration of Smad and MAPK pathways: a link and a linker revisited. Genes Dev. 2003;17:2993–7.
8. Kamaraju AK, Roberts AB. Role of Rho/ROCK and p38 MAP kinase pathways in transforming growth factor-beta-mediated Smad-dependent growth inhibition of human breast carcinoma cells in vivo. J Biol Chem. 2005;280:1024–36.
9. Matsuzaki K. Smad phospho-isoforms direct context-dependent TGF-beta signaling. Cytokine Growth Factor Rev. 2013;24:385–99.
10. Tripathi V, Sixt KM, Gao S, Xu X, Huang J, Weigert R, Zhou M, Zhang YE. Direct regulation of alternative splicing by SMAD3 through PCBP1 is essential to the tumor-promoting role of TGF-beta. Mol Cell. 2016;64:1010.
11. Shirakihara T, Horiguchi T, Miyazawa M, Ehata S, Shibata T, Morita I, Miyazono K, Saitoh M. TGF-β regulates isoform switching of FGF receptors and epithelial-mesenchymal transition. EMBO J. 2011;30:783–95.
12. Massague J, Seoane J, Wotton D. Smad transcription factors. Genes Dev. 2005;19:2783–810.
13. Lamouille S, Subramanyam D, Blelloch R, Derynck R. Regulation of epithelial-mesenchymal and mesenchymal-epithelial transitions by microRNAs. Curr Opin Cell Biol. 2013;25:200–7.
14. Piek E, Moustakas A, Kurisaki A, Heldin CH, ten Dijke P. TGF-(beta) type I receptor/ALK-5 and Smad proteins mediate epithelial to mesenchymal transdifferentiation in NMuMG breast epithelial cells. J Cell Sci. 1999;112(Pt 24):4557–68.
15. Sporn MB, Roberts AB. Transforming growth factor-beta: recent progress and new challenges. J Cell Biol. 1992;119:1017–21.
16. Moustakas A, Pardali K, Gaal A, Heldin CH. Mechanisms of TGF-beta signaling in regulation of cell growth and differentiation. Immunol Lett. 2002;82:85–91.
17. Schuster N, Krieglstein K. Mechanisms of TGF-beta-mediated apoptosis. Cell Tissue Res. 2002;307:1–14.
18. Bierie B, Moses HL. TGF-beta and cancer. Cytokine Growth Factor Rev. 2006;17:29–40.
19. Naito Y, Yoshioka Y, Yamamoto Y, Ochiya T. How cancer cells dictate their microenvironment: present roles of extracellular vesicles. Cell Mol Life Sci. 2017;74(4):697–713.
20. Barron DA, Rowley DR. The reactive stroma microenvironment and prostate cancer progression. Endocr Relat Cancer. 2012;19:R187–204.
21. Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. Nat Med. 2013;19:1423–37.
22. Miyazono K. Transforming growth factor-beta signaling in epithelial-mesenchymal transition and progression of cancer. Proc Jpn Acad Ser B Phys Biol Sci. 2009;85:314–23.
23. Saitoh M, Miyazawa K. Transcriptional and post-transcriptional regulation in TGF-beta-mediated epithelial-mesenchymal transition. J Biochem. 2012;151:563–71.
24. Greenburg G, Hay ED. Epithelia suspended in collagen gels can lose polarity and express characteristics of migrating mesenchymal cells. J Cell Biol. 1982;95:333–9.

25. Hay ED. An overview of epithelio-mesenchymal transformation. Acta Anat (Basel). 1995;154:8–20.
26. Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. Cell. 2009;139:871–90.
27. Fischer KR, Durrans A, Lee S, Sheng J, Li F, Wong ST, Choi H, El Rayes T, Ryu S, Troeger J, Schwabe RF, Vahdat LT, Altorki NK, Mittal V, Gao D. Epithelial-to-mesenchymal transition is not required for lung metastasis but contributes to chemoresistance. Nature. 2015;527:472–6.
28. Zheng X, Carstens JL, Kim J, Scheible M, Kaye J, Sugimoto H, Wu CC, Lebleu VS, Kalluri R. Epithelial-to-mesenchymal transition is dispensable for metastasis but induces chemoresistance in pancreatic cancer. Nature. 2015;527:525–30.
29. Zhou BP, Deng J, Xia W, Xu J, Li YM, Gunduz M, Hung MC. Dual regulation of Snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition. Nat Cell Biol. 2004;6(10):931–40.
30. Yang J, Dai C, Liu Y. A novel mechanism by which hepatocyte growth factor blocks tubular epithelial to mesenchymal transition. J Am Soc Nephrol. 2005;16(1):68–78.
31. Zheng H, Shen M, Zha YL, Li W, Wei Y, Blanco MA, Ren G, Zhou T, Storz P, Wang HY, Kang Y. PKD1 phosphorylation-dependent degradation of SNAIL by SCF-FBXO11 regulates epithelial-mesenchymal transition and metastasis. Cancer Cell. 2014;26(3):358–73.
32. Shirakihara T, Saitoh M, Miyazono K. Differential regulation of epithelial and mesenchymal markers by δEF1 proteins in epithelial mesenchymal transition induced by TGF-β. Mol Biol Cell. 2007;18:3533–44.
33. Funahashi J, Sekido R, Murai K, Kamachi Y, Kondoh H. Delta-crystallin enhancer binding protein delta EF1 is a zinc finger-homeodomain protein implicated in postgastrulation embryogenesis. Development. 1993;19(2):433–46.
34. Postigo AA. Opposing functions of ZEB proteins in the regulation of the TGFbeta/BMP signaling pathway. EMBO J. 2003;22(10):2443–52.
35. Llorens MC, Lorenzatti G, Cavallo NL, Vaglienti MV, Perrone AP, Carenbauer AL, Darling DS, Cabanillas AM. Phosphorylation regulates functions of ZEB1 transcription factor. J Cell Physiol. 2016;231(10):2205–17.
36. Moustakas A, Heldin CH. Mechanisms of TGFbeta-induced epithelial-mesenchymal transition. J Clin Med. 2016;5
37. Nieto MA, Huang RY, Jackson RA, Thiery JP. Emt: 2016. Cell. 2016;166:21–45.
38. Thuault S, Valcourt U, Petersen M, Manfioletti G, Heldin CH, Moustakas A. Transforming growth factor-beta employs HMGA2 to elicit epithelial-mesenchymal transition. J Cell Biol. 2006;174:175–83.
39. Kondo M, Suzuki H, Takehara K, Miyazono K, Kato M. Transforming growth factor-beta signaling is differentially inhibited by Smad2D450E and Smad3D407E. Cancer Sci. 2004;95:12–7.
40. Kowanetz M, Valcourt U, Bergstrom R, Heldin CH, Moustakas A. Id2 and Id3 define the potency of cell proliferation and differentiation responses to transforming growth factor beta and bone morphogenetic protein. Mol Cell Biol. 2004;24:4241–54.
41. Horiguchi K, Shirakihara T, Nakano A, Imamura T, Miyazono K, Saitoh M. Role of Ras signaling in the induction of snail by transforming growth factor-β. J Biol Chem. 2009;284:245–53.
42. Peinado H, Quintanilla M, Cano A. Transforming growth factor beta-1 induces snail transcription factor in epithelial cell lines: mechanisms for epithelial mesenchymal transitions. J Biol Chem. 2003;278:21113–23.
43. Saitoh M, Endo K, Furuya S, Minami M, Fukasawa A, Imamura T, Miyazawa K. STAT3 integrates cooperative Ras and TGF-beta signals that induce snail expression. Oncogene. 2016;35:1049–57.
44. Narimatsu M, Samavarchi-Tehrani P, Varelas X, Wrana JL. Distinct polarity cues direct Taz/Yap and TGFbeta receptor localization to differentially control TGFbeta-induced Smad signaling. Dev Cell. 2015;32:652–6.

45. Varelas X, Sakuma R, Samavarchi-Tehrani P, Peerani R, Rao BM, Dembowy J, Yaffe MB, Zandstra PW, Wrana JL. TAZ controls Smad nucleocytoplasmic shuttling and regulates human embryonic stem-cell self-renewal. Nat Cell Biol. 2008;10:837–48.
46. Tang Y, Feinberg T, Keller ET, Li XY, Weiss SJ. Snail/slug binding interactions with YAP/TAZ control skeletal stem cell self-renewal and differentiation. Nat Cell Biol. 2016;18:917–29.
47. Zhang H, von Gise A, Liu Q, Hu T, Tian X, He L, Pu W, Huang X, He L, Cai CL, Camargo FD, Pu WT, Zhou B. Yap1 is required for endothelial to mesenchymal transition of the atrioventricular cushion. J Biol Chem. 2014;289:18681–92.
48. Ozdamar B, Bose R, Barrios-Rodiles M, Wang HR, Zhang Y, Wrana JL. Regulation of the polarity protein Par6 by TGFbeta receptors controls epithelial cell plasticity. Science. 2005;307:1603–9.
49. Gunaratne A, Thai BL, Di Guglielmo GM. Atypical protein kinase C phosphorylates Par6 and facilitates transforming growth factor beta-induced epithelial-to-mesenchymal transition. Mol Cell Biol. 2013;33:874–86.
50. Kawata M, Koinuma D, Ogami T, Umezawa K, Iwata C, Watabe T, Miyazono K. TGF-beta-induced epithelial-mesenchymal transition of A549 lung adenocarcinoma cells is enhanced by pro-inflammatory cytokines derived from RAW 264.7 macrophage cells. J Biochem. 2012;151:205–16.
51. Ehata S, Hanyu A, Hayashi M, Aburatani H, Kato Y, Fujime M, Saitoh M, Miyazawa K, Imamura T, Miyazono K. Transforming growth factor-beta promotes survival of mammary carcinoma cells through induction of antiapoptotic transcription factor DEC1. Cancer Res. 2007;67:9694–703.
52. Shirakihara T, Kawasaki T, Fukagawa A, Semba K, Sakai R, Miyazono K, Miyazawa K, Saitoh M. Identification of integrin alpha3 as a molecular marker of cells undergoing epithelial-mesenchymal transition and of cancer cells with aggressive phenotypes. Cancer Sci. 2013;104:1189–97.
53. Bracken CP, Gregory PA, Kolesnikoff N, Bert AG, Wang J, Shannon MF, Goodall GJ. A double-negative feedback loop between ZEB1-SIP1 and the microRNA-200 family regulates epithelial-mesenchymal transition. Cancer Res. 2008;68:7846–54.
54. Lynch SM, O'Neill KM, McKenna MM, Walsh CP, McKenna DJ. Regulation of miR-200c and miR-141 by methylation in prostate cancer. Prostate. 2016;76:1146–59.
55. Siemens H, Jackstadt R, Hunten S, Kaller M, Menssen A, Gotz U, Hermeking H. miR-34 and SNAIL form a double-negative feedback loop to regulate epithelial-mesenchymal transitions. Cell Cycle. 2011;10:4256–71.
56. Zhang Z, Zhang B, Li W, Fu L, Fu L, Zhu Z, Dong JT. Epigenetic silencing of miR-203 upregulates SNAI2 and contributes to the invasiveness of malignant breast cancer cells. Genes Cancer. 2011;2:782–91.
57. Jin Y, Tymen SD, Chen D, Fang ZJ, Zhao Y, Dragas D, Dai Y, Marucha PT, Zhou X. MicroRNA-99 family targets AKT/mTOR signaling pathway in dermal wound healing. PLoS One. 2013;8:e64434.
58. Turcatel G, Rubin N, El-Hashash A, Warburton D. MIR-99a and MIR-99b modulate TGF-beta induced epithelial to mesenchymal plasticity in normal murine mammary gland cells. PLoS One. 2012;7:e31032.
59. Richards EJ, Zhang G, Li ZP, Permuth-Wey J, Challa S, Li Y, Kong W, Dan S, Bui MM, Coppola D, Mao WM, Sellers TA, Cheng JQ. Long non-coding RNAs (LncRNA) regulated by transforming growth factor (TGF) beta: LncRNA-hit-mediated TGFbeta-induced epithelial to mesenchymal transition in mammary epithelia. J Biol Chem. 2015;290:6857–67.
60. Yang S, Yao H, Li M, Li H, Wang F. Long non-coding RNA MALAT1 mediates transforming growth factor Beta1-induced epithelial-mesenchymal transition of retinal pigment epithelial cells. PLoS One. 2016;e0152687:11.
61. Yuan JH, Yang F, Wang F, Ma JZ, Guo YJ, Tao QF, Liu F, Pan W, Wang TT, Zhou CC, Wang SB, Wang YZ, Yang Y, Yang N, Zhou WP, Yang GS, Sun SH. A long noncoding RNA activated

by TGF-beta promotes the invasion-metastasis cascade in hepatocellular carcinoma. Cancer Cell. 2014;25:666–81.
62. Tay Y, Rinn J, Pandolfi PP. The multilayered complexity of ceRNA crosstalk and competition. Nature. 2014;505:344–52.
63. Warzecha CC, Sato TK, Nabet B, Hogenesch JB, Carstens RP. ESRP1 and ESRP2 are epithelial cell-type-specific regulators of FGFR2 splicing. Mol Cell. 2009;33:591–601.
64. Horiguchi K, Sakamoto K, Koinuma D, Semba K, Inoue A, Inoue S, Fujii H, Yamaguchi A, Miyazawa K, Miyazono K, Saitoh M. TGF-beta drives epithelial-mesenchymal transition through deltaEF1-mediated downregulation of ESRP. Oncogene. 2012;31:3190–201.
65. Ishii H, Saitoh M, Sakamoto K, Kondo T, Katoh R, Tanaka S, Motizuki M, Masuyama K, Miyazawa K. Epithelial splicing regulatory proteins 1 (ESRP1) and 2 (ESRP2) suppress cancer cell motility via different mechanisms. J Biol Chem. 2014;289:27386–99.
66. Warzecha CC, Jiang P, Amirikian K, Dittmar KA, Lu H, Shen S, Guo W, Xing Y, Carstens RP. An ESRP-regulated splicing programme is abrogated during the epithelial-mesenchymal transition. EMBO J. 2010;29:3286–300.
67. Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, Clark L, Bayani N, Coppe JP, Tong F, Speed T, Spellman PT, Devries S, Lapuk A, Wang NJ, Kuo WL, Stilwell JL, Pinkel D, Albertson DG, Waldman FM, McCormick F, Dickson RB, Johnson MD, Lippman M, Ethier S, Gazdar A, Gray JW. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell. 2006;10:515–27.
68. Hoshi Y, Endo K, Shirakihara T, Fukagawa A, Miyazawa K, Saitoh M. The potential role of regulator of G-protein signaling 16 in cell motility mediated by deltaEF1 family proteins. FEBS Lett. 2016;590:270–8.
69. Fukagawa A, Ishii H, Miyazawa K, Saitoh M. deltaEF1 associates with DNMT1 and maintains DNA methylation of the E-cadherin promoter in breast cancer cells. Cancer Med. 2015;4:125–35.
70. Ferrari-Amorotti G, Fragliasso V, Esteki R, Prudente Z, Soliera AR, Cattelani S, Manzotti G, Grisendi G, Dominici M, Pieraccioli M, Raschella G, Chiodoni C, Colombo MP, Calabretta B. Inhibiting interactions of lysine demethylase LSD1 with snail/slug blocks cancer cell invasion. Cancer Res. 2013;73:235–45.
71. Lin Y, Dong C, Zhou BP. Epigenetic regulation of EMT: the snail story. Curr Pharm Des. 2014;20:1698–705.
72. Zhou BP, Deng J, Xia W, Xu J, Li YM, Gunduz M, Hung MC. Dual regulation of Snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition. Nat Cell Biol. 2004;6(10):931–40.
73. Yang J, Dai C, Liu Y. A novel mechanism by which hepatocyte growth factor blocks tubular epithelial to mesenchymal transition. J Am Soc Nephrol. 2005;16(1):68–78.
74. Zheng H, Shen M, Zha YL, Li W, Wei Y, Blanco MA, Ren G, Zhou T, Storz P, Wang HY, Kang Y. PKD1 phosphorylation-dependent degradation of SNAIL by SCF-FBXO11 regulates epithelial-mesenchymal transition and metastasis. Cancer Cell. 2014;26(3):358–73.
75. Funahashi J, Sekido R, Murai K, Kamachi Y, Kondoh H. Delta-crystallin enhancer binding protein delta EF1 is a zinc finger-homeodomain protein implicated in postgastrulation embryogenesis. Development. 1993;19(2):433–46.
76. Postigo AA. Opposing functions of ZEB proteins in the regulation of the TGFbeta/BMP signaling pathway. EMBO J. 2003;22(10):2443–52.
77. Llorens MC, Lorenzatti G, Cavallo NL, Vaglienti MV, Perrone AP, Carenbauer AL, Darling DS, Cabanillas AM. Phosphorylation regulates functions of ZEB1 transcription factor. J Cell Physiol. 2016;231(10):2205–17.

Chapter 5
The Functional Interplay Between Pro-oncogenic RUNX2 and Hypoxia-Inducible Factor-1α (HIF-1α) During Hypoxia-Mediated Tumor Progression

Toshinori Ozaki, Mizuyo Nakamura, Takehiko Ogata, Meijie Sang, and Osamu Shimozato

Abstract Solid tumor tissues often have functional and phenotypical heterogeneities, arising at least in part from the local hypoxic tumor microenvironment (generally O_2 concentration is less than 2%). The elevated level of hypoxia is tightly associated with genetic instability, tumor progression, drug resistance, and/or poor clinical outcome after treatment, indicating that hypoxia exerts a strong selection pressure for the survival of cancer stem cells (CSCs) within tumors and also permits their maintenance. Thus, it has become urgent to precisely clarify the molecular basis of how hypoxia could contribute to the acquisition and/or maintenance of the aggressive phenotypes of this deadly disease. Meanwhile, cells keep genomic integrity to avoid genetic instability-mediated tumorigenesis through the proper stress response under normoxia. Upon hypoxia, hypoxia-inducible factor-1α (HIF-1α) which has an O_2-sensing ability accumulates and then facilitates tumor development through an induction of vascular endothelial growth factor (VEGF)-dependent angiogenesis. Therefore, the hypoxic HIF-1α/VEGF regulatory axis plays a vital role during the malignant tumor progression. Intriguingly, pro-oncogenic runt-related transcription factor 2 (RUNX2) has an ability to stimulate HIF-1α-mediated induction of VEGF. Recently, we have found for the first time that RUNX2 contributes to the acquisition of drug-resistant phenotype of malignant tumor cells. In this review, we focus on the functional interplay between HIF-1α/VEGF and RUNX2 within the hypoxic tumor microenvironment. Finally, we would like to discuss the potential therapeutic strategy targeting this tumor hypoxia.

T. Ozaki (✉) • M. Nakamura • T. Ogata • O. Shimozato
Laboratory of DNA Damage Signaling, Chiba Cancer Center Research Institute, Chiba, Japan
e-mail: tozaki@chiba-cc.jp

M. Sang
Laboratory of DNA Damage Signaling, Chiba Cancer Center Research Institute, Chiba, Japan

Department of Regenerative Medicine, Graduate School of Medicine and Pharmaceutical Science, University of Toyama, Toyama, Japan

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N. Shinomiya et al. (eds.), *Regulation of Signal Transduction in Human Cell Research*, Current Human Cell Research and Applications,
https://doi.org/10.1007/978-981-10-7296-3_5

Keywords Cancer stem cells • Drug resistance • HIF-1α • Hypoxia • RUNX2 • VEGF

5.1 Introduction

Accumulating evidence strongly suggests that tumor cell microenvironments including hypoxia play a pivotal role in the acquisition and/or maintenance of malignant phenotypes of advanced tumors. Although tumor cells with a higher proliferation rate consume a large amount of oxygen and nutrients, they are exposed to a serious hypoxic condition. To survive under these severe conditions, hypoxic response takes place within tumors [1, 2].

One of the initial molecular events in response to hypoxia is the stabilization and activation of hypoxia-inducible factor-1α (HIF-1α). HIF-1α, which recognizes and binds to HRE (hypoxia-responsive element) within its target gene promoter/enhancer, is a basic helix-loop-helix family of transcription factor. Under normoxia, the amount of HIF-1α is kept at an extremely low level within cell nucleus through von Hippel-Lindau (VHL) E3 ubiquitin ligase-mediated proteasomal degradation system [3]. Upon hypoxia, VHL is dissociated from HIF-1α, and thereby HIF-1α becomes stable without proteolytic degradation. Stabilized and activated HIF-1α then induces the expression of its numerous target gene products such as pro-angiogenic vascular endothelial growth factor (VEGF), erythropoietin, and enolase [4, 5].

As expected, VEGF plays a central role in hypoxic response to assist the survival of a certain population of tumor cells exposed to serious hypoxic conditions. VEGF promotes the formation of new blood vessels around hypoxic areas, which supply hypoxic tumor cells with the enough amount of oxygen as well as nutrients, and then a certain subset of tumor cells acquires much more malignant phenotypes including the enhanced drug resistance and the increased metastatic potential [6–9]. Indeed, it has been shown that the aberrant overexpression of *VEGF* is closely associated with poor clinical prognosis of the patients with a variety of aggressive tumors [10, 11]. With these in mind, HIF-1α/VEGF regulatory axis in response to hypoxia has been considered to be one of the promising molecular targets for cancer therapy [12].

The evolutionarily conserved small noncoding RNAs, termed microRNAs (miRNAs), regulate their target gene expression primarily through the posttranscription level in a sequence-specific manner [13]. Of interest, increasing evidence strongly indicates that miRNAs are implicated in the regulation of various cellular processes such as hypoxic response [14]. For example, it has been described that miR-622 directly targets *HIF-1*α and then significantly impedes HIF-1α-mediated tumor cell migration as well as invasion in response to hypoxia [15]. On the other hand, miR-630, a HIF-1α-induced miRNA, contributed to the promotion of tumor growth and metastasis [16]. Thus, it is likely that a certain set of miRNAs involved in hypoxic response might serve as critical prognostic indicators and also potential molecular targets for future cancer therapy.

Runt-related transcription factor 2 (RUNX2) is one of the RUNX family members composed of RUNX1, RUNX2, and RUNX3. It has been well established that RUNX2 is a master regulator of osteoblast differentiation and bone formation. *RUNX2*-deficient mice displayed a complete loss of bone formation [17, 18]. Consistent with these observations, RUNX2 transactivates a variety of its target genes implicated in osteogenesis such as *type I collagen*, *osteopontin*, and *osteocalcin* [19]. However, this previous point of view has been challenged by the findings showing that, in addition to osteogenesis, RUNX2 has a strong oncogenic potential. Firstly, it has been shown that aberrant overexpression of *RUNX2* is detectable in numerous tumor tissues [20–22]. Secondly, RUNX2 has an ability to transactivate several tumor cell invasion-related genes including *MMP-9* and *MMP-13* [23, 24]. Lastly, we have found that depletion of *RUNX2* significantly improves drug sensitivity of various tumor cells ([25, 26]).

In the present review article, we describe the basic background of hypoxic response of tumor cells and then discuss the potential therapeutic strategies which might overcome hypoxic response-mediated malignant progression of tumor cells based on our current observations.

5.2 Hypoxia-Inducible Factor-1 (HIF-1) and Hypoxic Response of Tumor Cells

To survive, the majority of solid tumor cells exhaust a lot of nutrients and oxygen provided from the surrounding normal vasculature, and thereby tumor tissues become hypoxic (insufficient oxygen levels). Hypoxic tumors appear to become much more aggressive (the reduced cell death, the enhanced drug resistance, the higher resistance to radiotherapy, and the increased metastatic potential) [27–30]. Consistent with these observations, tumor hypoxia is an independent poor prognostic factor for the survival of tumor patients irrespective of treatment modality [31, 32].

To increase their mass under this serious hypoxic condition, tumor cells require the additional nutrients and oxygen. The angiogenesis (the formation of new blood vessels) which plays a pivotal role in malignant tumor progression as well as metastasis is a multistage biological process tightly regulated by a balance between pro-angiogenic and anti-angiogenic signalings [33, 34]. The tumor angiogenesis, which is one of the hallmarks of the hypoxic tumors, is implicated in the accelerated proliferation rate of the vascular endothelial cells [8].

The vascular endothelial growth factor (VEGF) (also known as VEGF-A), which is the most important angiogenic secreted dimeric glycoprotein, promotes these angiogenic processes such as the vascular endothelial cell proliferation and the development of the tumor vessels under the hypoxic condition [35]. As described [36], VEGF family is composed of several structurally and functionally related proteins including VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and placental growth factor. Among them, VEGF-A has been the most studied member of VEGF family. Since the secretion of VEGF from the hypoxic tumor cells has

been shown to trigger the advanced tumor development [6, 7, 9], VEGF-mediated formation of tumor vessels is one of the essential hypoxic responses of tumor cells. Therefore, preventing these processes might contribute at least in part to prohibit both aggressive tumor progression and metastasis [10, 11].

Transcriptionally active hypoxia-inducible factor-1 (HIF-1), a basic helix-loop-helix family of transcription factor, is a heterodimer made up of α- and β-subunits. Under normal conditions, HIF-1α is continuously produced and simultaneously degraded through ubiquitin-proteasome breakdown system driven by von Hippel-Lindau (VHL) E3 ubiquitin ligase, whereas HIF-1β is constitutively expressed and kept at constant level within cell nucleus [3]. The immediate molecular response to hypoxia is stabilization of HIF-1α. Upon hypoxia or loss of functional VHL, HIF-1α is stabilized, forms a heterodimeric complex with HIF-1β, and binds to hypoxia-responsive elements (HREs) within its target gene promoters to trigger a concerted transcriptional response [5]. Among HIF-1-target genes, *VEGF* is the extensively studied HIF-1-regulated gene [4]. HIF-1-mediated deregulated expression of VEGF leads to the development of hypoxic tumors through the promotion of the angiogenesis as mentioned above. As expected, the aberrant expression of VEGF has been shown to be associated with the poor prognosis of various types of human tumors [37, 38] (Fig. 5.1). Therefore, prohibition of VEGF-mediated pro-angiogenic pathway improved the efficacy of various anticancer drugs on breast, cervix, stomach, lung, colon, rectum, and ovary carcinomas [39–41].

5.3 Maintenance and Propagation of Cancer Stem Cells (CSCs) Under Hypoxia

It has been well known that phenotypic and functional heterogeneity is a common property of a variety of solid tumors, arising at least in part from tumor cell microenvironments. According to cancer stem cell (CSC) theory, which might recapitulate the primary tumors, only a small subset of tumor cell population has a strong pro-oncogenic potential. A subset of tumor cells which expresses cell surface normal stem cell markers such as CD24 and CD44 has been shown to be much more tumorigenic [42]. In support of these findings, it has been described that malignant glioma and colon cancer include CSCs [43, 44]. Growing evidence indicates that CSCs cause the acquisition of malignant phenotypes of advanced tumors including drug resistance and recurrence after therapy [45].

Since hypoxia is one of the critical properties of tumor cell microenvironments, it is possible that hypoxia-induced HIF-1α might contribute to the maintenance and/or survival of CSCs. Bos et al. demonstrated that the expression level of HIF-1α protein in breast cancer tissues is larger in poorly differentiated than well-differentiated lesions, indicating that HIF-1α plays a role in the maintenance of the undifferentiated state of aggressive tumors [46]. Soeda et al. found that hypoxia promotes cell survival of undifferentiated CD133-positive glioma-initiating CSCs through the activation of HIF-1α, whereas CSC differentiation is markedly

prohibited under hypoxia [47]. CD133 has been considered to be one of the molecular markers of CSC population [48]. Based on their results, depletion of HIF-1α led to a massive reduction in the sphere-forming ability of glioma CSCs. Collectively, CSCs are obviously dependent on HIF-1α for their survival, self-renewal, and tumor growth.

Moreover, Heddleston et al. found that hypoxia increases number of CSC population and enhances the stem-like phenotype of the established tumor cell lines [49]. Similarly, Dong et al. described that hypoxia potentiates CSC sphere formation and causes an increase in number of CD44-positive colon cancer CSC subpopulations [50]. From their observations, the expression level of inhibitor of DNA-binding protein 2 (Id2) in colon cancer CSCs was significantly elevated in response to hypoxia through the activation of pro-oncogenic Wnt/β-catenin signaling pathway. Consistently, silencing of *Id2* markedly attenuated hypoxia-mediated CSC sphere

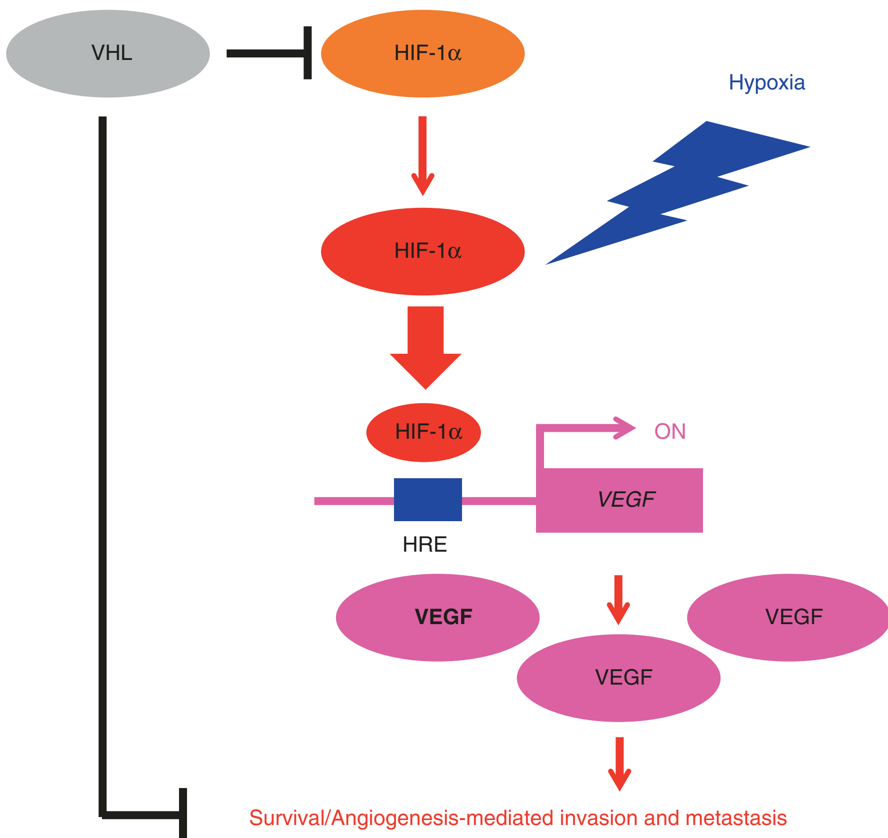

Fig. 5.1 HIF-1α is a critical determinant for hypoxic response. Upon hypoxia, HIF-1α becomes stabilized and transactivates its target genes including *VEGF*. VEGF-mediated angiogenesis contributes to the acquisition and/or maintenance of malignant phenotypes of aggressive tumors

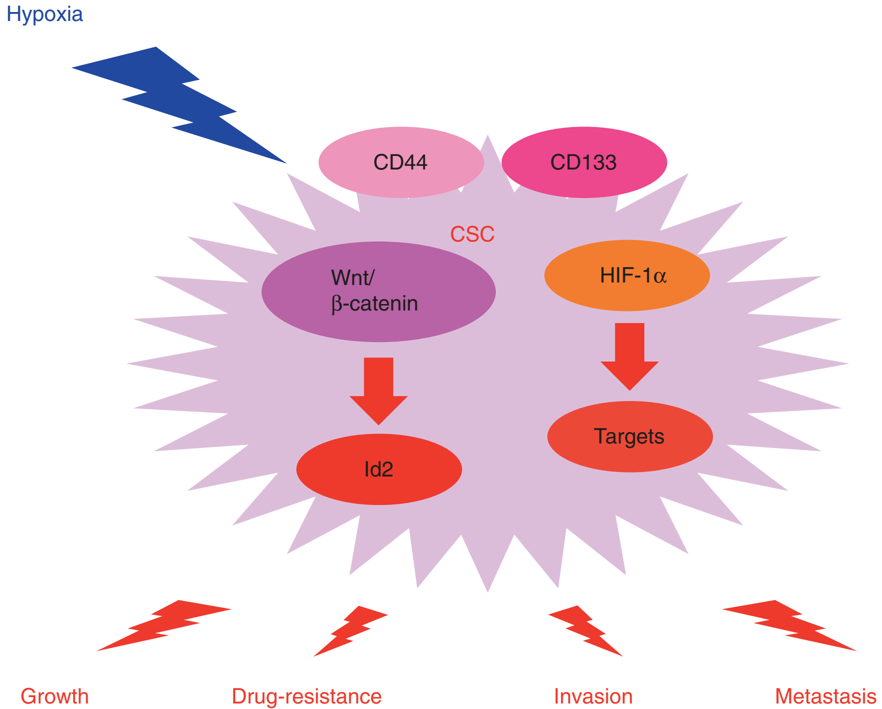

Fig. 5.2 Molecular basis of hypoxic response of cancer stem cells (CSCs). When CD44-/CD133-positive cancer stem cells (CSCs) within tumors are exposed to hypoxia, in addition to HIF-1α, Wnt/β-catenin pro-oncogenic pathway becomes activated and promotes Id2-mediated tumorigenesis

formation and also tumor metastasis in vivo. Intriguingly, highly aggressive pancreatic and colon cancer cells expressed a large amount of Id2 [51, 52].

Together, in addition to HIF-1α, Wnt/β-catenin-dependent augmentation of Id2 might be involved in the maintenance and/or survival of CSCs in response to hypoxia (Fig. 5.2).

5.4 Regulatory Role of MicroRNAs in Response to Hypoxia

MicroRNAs (miRNAs) are evolutionarily conserved small noncoding regulatory RNAs of around 22 nucleotides in length, which recognize and bind to the 3′-untranslated regions (3′-UTR) of their target mRNAs, repressing their translation [13]. Bioinformatic analysis indicates that more than 30% of protein-coding genes might be regulated by miRNAs [53]. A growing body of evidence strongly suggests that numerous siRNAs act as tumor suppressors or oncogene products. For example, Cheng et al. demonstrated that miR-622 has an ability to downregulate HIF-1α

and then prohibits metastatic spread of lung cancer in mouse xenograft model [15]. Xue et al. found that hypoxia-mediated repression of miR-15-16, which targets angiogenic *FGF2*, promotes tumor angiogenesis and metastasis [54].

In contrast, Rupaimoole et al. found that miR-630 reduces the expression level of Dicer, which is involved in miRNA biogenesis, and stimulates tumor growth as well as metastasis [16]. Ge et al. described that miR-421 targets *E-cadherin* as well as *caspase-3* and then stimulates metastasis [55]. According to their results, the expression level of miR-421 was significantly higher in advanced gastric cancer tissues than localized ones. Similarly, Devlin et al. revealed that miR-210 is upregulated in response to hypoxia and contributes to tumor metastasis [56]. In addition, miR-382 has been shown to be angiogenic miRNA targeting tumor suppressor PTEN (phosphatase and tensin homolog) [57]. Since the expression of miR-630, miR-421, miR-210, and miR-382 has been shown to be regulated by HIF-1α, it is worth noting that a certain subset of miRNAs responsible for hypoxia-induced tumor invasion/metastasis participates in HIF-1α-mediated oncogenic pathway. In this connection, several miRNAs implicated in hypoxic response might provide potential therapeutic clues to overcome malignant phenotypes of advanced tumors.

5.5 Pro-oncogenic Property of Runt-Related Transcription Factor 2 (RUNX2)

Runt-related transcription factor 2 (RUNX2) is a nuclear sequence-specific transcription factor responsible for the induction of bone formation and osteoblast differentiation. Indeed, *RUNX2*-deficient mice died just after birth and exhibited a complete loss of bone formation [17, 18]. In support of these observations, RUNX2 transactivates a number of osteogenic indicators such as type I collagen, osteopontin, and osteocalcin through RUNX2-responsive elements within their promoter regions [19].

Meanwhile, accumulating evidence demonstrated that *RUNX2* is highly expressed in a variety of tumor tissues as compared to their corresponding normal ones. For example, Pratap et al. described that *RUNX2* is aberrantly overexpressed in breast and prostate cancers [21]. Kayed et al. demonstrated that pancreatic ductal adenocarcinoma tissues highly express *RUNX2* as compared to the normal pancreas [20]. In addition, Wang et al. revealed that the expression level of *RUNX2* is higher in ovarian cancer tissues than normal ovarian ones [22]. Lastly, Boregowda et al. found that RUNX2 is highly expressed in melanoma tissues relative to melanocytes [58]. Accordingly, it has been shown that the expression level of *RUNX2* is employed as a prognostic indicator of non-small cell lung carcinoma patients [59]. These observations imply that, in addition to osteoblast differentiation and bone formation, RUNX2 has a strong pro-oncogenic potential in vivo.

Recently, we have found for the first time that RUNX2 is tightly linked to the drug-resistant phenotype of malignant tumor-derived cells such as osteosarcoma U2OS cells and pancreatic cancer AsPC-1 cells ([25], [26]). The drug resistance has been considered to be one of the hallmarks of advanced tumors [30]. From our observations, siRNA-mediated knockdown of *RUNX2* remarkably improved adriamycin (ADR) and gemcitabine (GEM) sensitivity of U2OS and AsPC-1 cells, respectively.

5.6 Functional Implication of RUNX2 in the Regulation of the Hypoxic Response

Since RUNX2 has a pro-oncogenic function, it is conceivable that RUNX2 might be closely implicated in the regulation of hypoxic response. Of interest, Pratap et al. described that RUNX2 transactivates tumor cell invasion-related *MMP-9* gene in bone metastatic tumor cells [24]. Similarly, Mendoza-Villanueva et al. found that RUNX2 directly regulates the expression of *MMP-13* and promotes the malignant invasion of breast cancer cells [23]. Forced expression of RUNX2 in prostate cancer cells stimulated their invasiveness [60]. In accordance with these observations, depletion of *RUNX2* resulted in a significant reduction of migration and invasion rate of colon cancer cells [61]. Furthermore, it has been described that pro-oncogenic PI3K/AKT pathway directly or indirectly augments the expression and the transcriptional activity of RUNX2, while RUNX2 also stimulates PI3K/AKT pathway, indicating that this positive feedback loop regulatory mechanism is one of the major driving forces during the malignant tumor cell progression [62]. Thus, it is possible that RUNX2 participates in the acquisition of the malignant phenotypes of the aggressive tumors such as invasion and metastasis.

Consistent with the above-mentioned findings, it has been also shown that RUNX2 has an ability to enhance *VEGF* transcription [63]. Of note, Lee et al. revealed that RUNX2 stabilizes HIF-1α through the inhibition of VHL-mediated ubiquitination of HIF-1α and stimulates the transcriptional activity of HIF-1α [64]. Kwon et al. reported that RUNX2 forms a complex with HIF-1α in cell nucleus and is then efficiently recruited onto *VEGF* promoter region [65]. Additionally, it has been described that hypoxia-mediated induction of RUNX2 drives the malignant progression of numerous tumors through the direct upregulation of anti-apoptotic Bcl-2 [66]. Together, it is likely that RUNX2 potentiates HIF-1α and thus promotes angiogenesis-mediated tumor cell invasion and/or metastasis in response to hypoxia (Fig. 5.3).

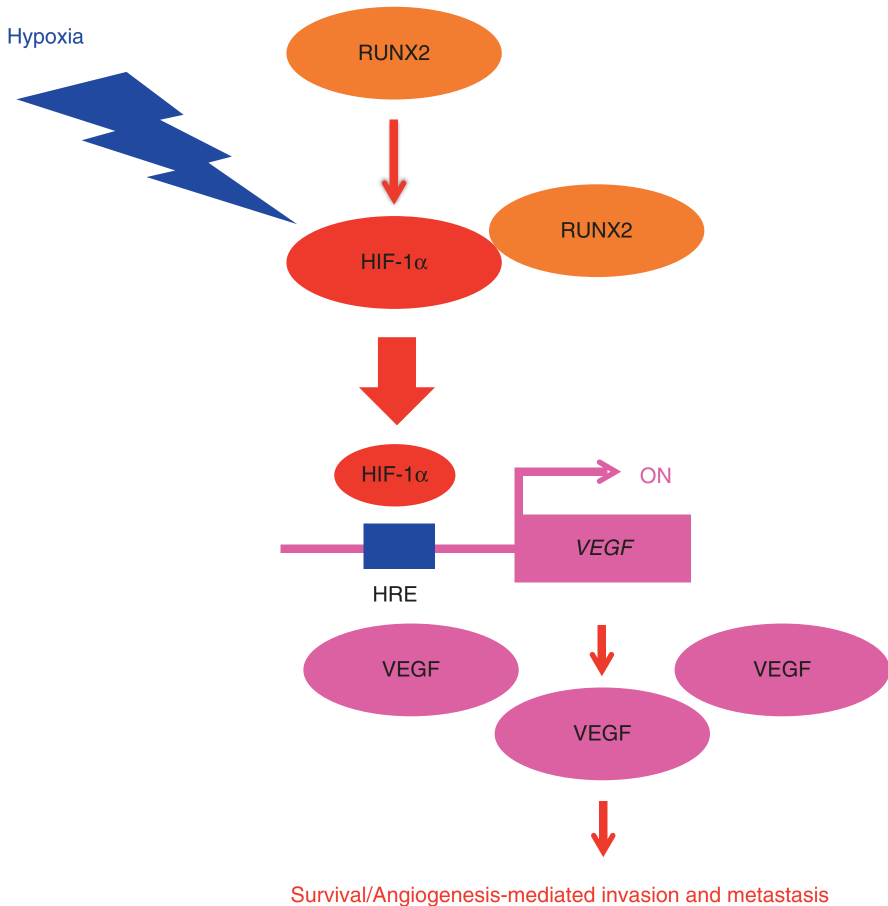

Fig. 5.3 RUNX2 augments HIF-1α-dependent hypoxic response. RUNX2 directly interacts with HIF-1α, enhances its sequence-specific transactivation ability, and thereby augments HIF-1α-dependent hypoxic response

5.7 Future Therapeutic Strategy Targeting Hypoxic Response

As described above, hypoxia-dependent tumor angiogenesis plays a critical role in the promotion of invasion and/or metastasis. These findings prompted us to develop anti-tumor drugs which block HIF-1α-/VEGF-induced angiogenesis. To our

knowledge, a number of VEGF-targeting drugs have been produced and approved for clinical treatment. For example, bevacizumab, a monoclonal antibody against VEGF-A, has been approved for the treatment of the patients with renal cancer [67]. In addition to VEGF, blocking VEGF receptor (VEGFR)-mediated signaling might be a promising approach to develop anti-tumor drugs. Recently, Li et al. described that a small chemical compound termed DW10075 selectively prohibits kinase activity of VEGFR [68]. However, chemotherapeutic drugs targeting HIF-1α/VEGF/VEGFR have limited efficacy against malignant tumors and sometimes cause adverse effects [69].

Given that RUNX2 further stimulates HIF-1α-mediated induction of VEGF [63], it is highly possible that depletion of *RUNX2* suppresses an aggressive progression of malignant tumors through the downregulation of VEGF. Intriguingly, several lines of evidence imply that certain miRNAs might regulate the expression of *RUNX2*. For example, the diminished expression of miR-135 or miR-203 led to the enhancement of *RUNX2* expression in metastatic breast cancer cells [70]. According to their results, miR-135 and miR-203 were highly expressed in normal breast epithelial cells, whereas *RUNX2* was undetectable. In contrast, metastatic breast cancer tissues expressed *RUNX2* but not miR-135 or miR-203.

Similarly, exogenous expression of miR-34c in osteosarcoma cells decreased the expression level of *RUNX2* [71]. Thus, it is likely that miRNA-mediated downregulation of *RUNX2* causes the efficient suppression of malignant phenotypes of the aggressive tumors.

Alternatively, we have found that siRNA-mediated silencing of *RUNX2* improves the efficacy of anti-tumor drugs [25, 26]. Unfortunately, it has been well known that siRNA is extremely unstable and thus its effect is transient. To overcome this weakness, Zorde Khvalevsky et al. developed a local prolonged siRNA delivery system (termed LODER) [72]. According to their results, LODER system blocked the degradation of siRNA and released intact siRNA slowly into tumor cells over a few months. Collectively, it is suggestive that LODER system overcomes the present siRNA delivery obstacles, and thus siRNA-mediated knockdown of *RUNX2* by employing LODER as a delivery system is an attractive therapeutic strategy for the treatment of the patients with malignant tumors.

5.8 Conclusion

A growing body of evidence implies that tumor microenvironments composed of several cell populations including tumor cells play vital roles in the regulation of the malignant tumor progression under severe hypoxic condition. Hypoxia-dependent stabilization and nuclear access of HIF-1α stimulate the expression of VEGF, which contributes to tumor cell survival, invasion, and metastasis through the formation of new blood vessels. Meanwhile, RUNX2 is associated with HIF-1α and further potentiates its sequence-specific transactivation ability. Thus, RUNX2/HIF-1α

regulatory axis is essential for VEGF-mediated malignant tumor progression in response to hypoxia, and RUNX2 might be one of the attractive molecular targets for cancer therapy.

Acknowledgments The authors are grateful to Dr. Hiroki Nagase for his helpful discussions.

Conflicts of Interest The authors have no conflict of interest.

References

1. Biddlestone J, Bandarra D, Rocha S. The role of hypoxia in inflammatory disease. Int J Mol Med. 2015;35:859–69.
2. Span PN, Bussink J. Biology of hypoxia. Sem Nucl Med. 2015;45:101–9.
3. Kaelin WG Jr. Treatment of kidney cancer: insights provided by the VHL tumor-suppressor protein. Cancer. 2009;(115):2262–72.
4. Forsythe JA, et al. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol. 1996;16:4604–13.
5. Keith B, Johnson RS, Simon MC. HIF1α and HIF2α: sibling rivalry in hypoxic tumour growth and progression. Nat Rev Cancer. 2012;12:9–22.
6. Claffey KP, Robinson GS. Regulation of VEGF/VPF expression in tumor cells: consequences for tumor growth and metastasis. Cancer Metastasis Rev. 1996;15:165−176.
7. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med. 2003;9:669−676.
8. Hu K, et al. Hypoxia-inducible factor 1 upregulation of both VEGF and ANGPTL4 is required to promote the angiogenic phenotype in uveal melanoma. Oncotarget. 2016;7(7):7816–28. 10.18632/oncotarget.6868.
9. Price DJ, et al. Role of vascular endothelial growth factor in the stimulation of cellular invasion and signaling of breast cancer cells. Cell Growth Differ. 2001;12:129–−135.
10. Grothey A, Galanis E. Targeting angiogenesis: progress with anti-VEGF treatment with large molecules. Nat Rev Clin Oncol. 2009;6:507−518.
11. Kerbel R, Folkman J. Clinical translation of angiogenesis inhibitors. Nat Rev Cancer. 2002;2:727–39.
12. Ulivi P, Marisi G, Passardi A. Relationship between hypoxia and response to antiangiogenic therapy in metastatic colorectal cancer. Oncotarget. 2016;7:46678–91. 10.18632/oncotarget.8712.
13. Shivdasani RA. MicroRNAs: regulators of gene expression and cell differentiation. Blood. 2006;108:3646–53.
14. Zhao Y, et al. miRNA-directed regulation of VEGF in tilapia under hypoxia condition. Biochem Biophys Res Commun. 2014;454(1):183–8.
15. Cheng S, et al. Global microRNA depletion suppresses tumor angiogenesis. Genes Dev. 2014;28:1054–67.
16. Rupaimoole R, et al. Hypoxia-upregulated microRNA-630 targets Dicer, leading to increased tumor progression. Oncogene. 2016;35(33):4312–20. https://doi.org/10.1038/onc.2015.492.
17. Komori T, et al. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell. 1997;89:755–64.
18. Otto F, et al. Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell. 1997;89:765–71.
19. Karsenty G. The genetic transformation of bone biology. Genes Dev. 1999;13:3037–51.
20. Kayed H. Regulation and functional role of the Runt-related transcription factor-2 in pancreatic cancer. Br J Cancer. 2007;97(8):1106–15.

21. Pratap J, et al. Regulatory roles of Runx2 in metastatic tumor and cancer cell interactions with bone. Cancer Metastasis Re. 2006;25(4):589–600.
22. Wang ZQ, et al. Inhibition of RUNX2 transcriptional activity blocks the proliferation, migration and invasion of epithelial ovarian carcinoma cells. PLoS One. 2013;8(10):e74384.
23. Mendoza-Villanueva D, et al. The Runx transcriptional co-activator, CBFbeta, is essential for invasion of breast cancer cells. Mol Cancer. 2010;9:171.
24. Pratap J, et al. The Runx2 osteogenic transcription factor regulates matrix metalloproteinase 9 in bone metastatic cancer cells and controls cell invasion. Mol Cell Biol. 2005;25(19):8581–91.
25. Ozaki T, et al. Runt-related transcription factor 2 (RUNX2) inhibits p53-dependent apoptosis through the collaboration with HDAC6 in response to DNA damage. Cell Death Dis. 2013;4:e610.
26. Sugimoto K, et al. Silencing of RUNX2 enhances gemcitabine sensitivity of p53-deficient human pancreatic cancer AsPC-1 cells through the stimulation of TAp63-mediated cell death. Cell Death Dis. 2015;6:e1914.
27. Carlson DJ, Yenice KM, Orton CG. Tumor hypoxia is an important mechanism of radioresistance in hypofractionated radiotherapy and must be considered in the treatment planning process. Med Phys. 2011;38(12):6347–50.
28. Graeber TG, et al. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature. 1996;379:88–91.
29. Subarsky P, Hill RP. The hypoxic tumour microenvironment and metastatic progression. Clin Exp Metastasis. 2003;20:237–50.
30. Wartenberg M, et al. Regulation of the multidrug resistance transporter P-glycoprotein in multicellular tumor spheroids by hypoxia-inducible factor (HIF-1) and reactive oxygen species. FASEB J. 2003;17:503–5.
31. McKeown SR. Defining normoxia, physoxia and hypoxia in tumours-implications for treatment response. Br J Radiol. 2014;87:20130676.
32. Nordsmark M, Overgaard J. Tumor hypoxia is independent of hemoglobin and prognostic for loco-regional tumor control after primary radiotherapy in advanced head and neck cancer. Acta Oncol. 2004;43(4):396–403.
33. McDougall SR, Anderson ARA, Chaplain MAJ. Mathematical modelling of dynamic adaptive tumour-induced angiogenesis: clinical implications and therapeutic targeting strategies. J Theor Biol. 2006;7:564–89.
34. Nishida N, et al. Angiogenesis in cancer. Vasc Health Risk Manag. 2006;2:213–9.
35. Ellis LM, Hicklin DJ. VEGF-targeted therapy: mechanisms of anti-tumour activity. Nat Rev Cancer. 2008;8:579−591.
36. Otrock ZK, Makarem JA, Shamseddine AI. Vascular endothelial growth factor family of ligands and receptors: review. Blood Cells Mol Dis. 2007;38(3):258–68.
37. Hirayama N, et al. Pleural effusion VEGF levels as a prognostic factor of malignant pleural mesothelioma. Respir Med. 2011;105:137–42.
38. Hsu IL, et al. Angiogenetic biomarkers in non-small cell lung cancer with malignant pleural effusion: Correlations with patient survival and pleural effusion control. Lung Cancer. 2009;65:371–6.
39. Blagosklonny MV. Antiangiogenic therapy and tumor progression. Cancer Cell. 2004;5:13–7.
40. Bottsford-Miller JN, Coleman RL, Sood AK. Resistance and escape from antiangiogenesis therapy: clinical implications and future strategies. J Clin Oncol. 2012;30:4026–34.
41. Jayson GC, Hicklin DJ, Ellis LM. Antiangiogenic therapy-evolving view based on clinical trial results. Nat Rev Clin Oncol. 2012;9:297–303.
42. Al-Hajj M, et al. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A. 2003;100:3983–8.
43. Ricci-Vitiani L, et al. Identification and expansion of human colon-cancer-initiating cells. Nature. 2007;445:111–5.
44. Singh SK, et al. Identification of a cancer stem cell in human brain tumors. Cancer Res. 2003;63:5821–8.

45. Visvader JE, Lindeman GJ. Cancer stem cells: current status and evolving complexities. Cell Stem Cell. 2012;10(6):717–28.
46. Bos R, et al. Levels of hypoxia-inducible factor-1 alpha during breast carcinogenesis. J Natl Cancer Inst. 2001;93(4):309–14.
47. Soeda et al. Hypoxia promotes expansion of the CD133-positive glioma stem cells through activation of HIF-1α. Oncogene. 2009;28:3949–59.
48. Jung P, et al. Isolation and in vitro expansion of human colonic stem cells. Nat Med. 2011;17:1225–7.
49. Heddleston JM, et al. Hypoxia inducible factors in cancer stem cells. Br J Cancer. 2010;102(5):789–95.
50. Dong et al. The Wnt/β-catenin signaling/Id2 cascade mediates the effects of hypoxia on the hierarchy of colorectal-cancer stem cells. Sci Rep. 2016;6:22966.
51. Benezra R, Rafii S, Lyden D. The Id proteins and angiogenesis. Oncogene. 2001;20(58):8334–41.
52. Kleeff J, et al. The helix-loop-helix protein Id2 is overexpressed in human pancreatic cancer. Cancer Res. 1998;58:3769–72.
53. Drakaki A, Iliopoulos D. MicroRNA gene networks in oncogenesis. Curr Genomics. 2009;10:35–41.
54. Xue G, et al. c-Myc-mediated repression of miR-15-16 in hypoxia is induced by increased HIF-2α and promotes tumor angiogenesis and metastasis by upregulating FGF2. Oncogene. 2015;34(11):1393–406.
55. Ge X, et al. MicroRNA-421 regulated by HIF-1α promotes metastasis, inhibits apoptosis, and induces cisplatin resistance by targeting E-cadherin and caspase-3 in gastric cancer. Oncotarget. 2016;7(17):24466–82. 10.18632/oncotarget.
56. Devlin C, et al. miR-210: More than a silent player in hypoxia. IUBMB Life. 2011;63:94–100.
57. Seok JK, et al. MicroRNA-382 induced by HIF-1alpha is an angiogenic miR targeting the tumor suppressor phosphatase and tensin homolog. Nucleic Acids Res. 2014;42:8062–72.
58. Boregowda RK, et al. RUNX2 is overexpressed in melanoma cells and mediates their migration and invasion. Cancer Lett. 2014;348(1–2):61–70.
59. Li H, et al. Clinical significance of RUNX2 expression in patients with nonsmall cell lung cancer: a 5-year follow-up study. Tumour Biol. 2013;34(3):1807–12.
60. Baniwal SK, et al. Runx2 transcriptome of prostate cancer cells: insights into invasiveness and bone metastasis. Mol Cancer. 2010;9:258.
61. Sase T, et al. Runt-related transcription factor 2 in human colon carcinoma: a potent prognostic factor associated with estrogen receptor. Int J Cancer. 2012;131:2284–93.
62. Cohen-Solal KA, Boregowda RK, Lasfar A. RUNX2 and the PI3K/AKT axis reciprocal activation as a driving force for tumor progression. Mol Cancer. 2015;14:137.
63. Zelzer E, et al. Tissue specific regulation of VEGF expression during bone development requires Cbfa1/Runx2. Mech Dev. 2001;106:97–106.
64. Lee SH, et al. Runx2 protein stabilizes hypoxia-inducible factor-1α through competition with von Hippel-Lindau protein (pVHL) and stimulates angiogenesis in growth plate hypertrophic chondrocytes. J Biol Chem. 2012;287(18):14760–71.
65. Kwon TG, et al. Physical and functional interactions between Runx2 and HIF-1α induce vascular endothelial growth factor gene expression. J Cell Biochem. 2011;112(12):3582–93.
66. Browne G, et al. Bicalutamide-induced hypoxia potentiates RUNX2-mediated Bcl-2 expression resulting in apoptosis resistance. Br J Cancer. 2012;107(10):1714–21.
67. Willett CG, et al. Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer. Nat Med. 2004;10:145–7.
68. Li MY, et al. DW10075, a novel selective and small-molecule inhibitor of VEGFR, exhibits antitumor activities both *in vitro* and *in vivo*. Acta Pharmacol Sin. 2016;37(3):398–407.
69. Zhang Y, et al. The DEK oncogene activates VEGF expression and promotes tumor angiogenesis and growth in HIF-1α-dependent and -independent manners. Oncotarget. 2016;7(17):23740–56. 10.18632/oncotarget.8060.

70. Taipaleenmäki H, et al. Targeting of Runx2 by miRNA-135 and miRNA-203 impairs progression of breast cancer and metastatic bone disease. Cancer Res. 2015;75(7):1433–44.
71. van der Deen M. MicroRNA-34c inversely couples the biological functions of the runt-related transcription factor RUNX2 and the tumor suppressor p53 in osteosarcoma. J Biol Chem. 2013;288(29):21307–19.
72. Zorde Khvalevsky E, et al. Mutant KRAS is a druggable target for pancreatic cancer. Proc Natl Acad Sci U S A. 2013;100:20723–8.

Chapter 6
DNA Damage: Cellular Responses, Repair, and Cancer Treatment

Brian M. Cartwright, Phillip R. Musich, and Yue Zou

Abstract The maintenance of genomic stability in the face of endogenous and exogenous sources of DNA damage requires a robust and comprehensive cellular response. This response, appropriately deemed the DNA damage response (DDR), facilitates changes in the cellular environment promoting and coordinating cell cycle arrest, DNA repair, and cell death in cases of extreme or prolonged genomic insult. Initiation of DDR is primarily elicited by three members of the PIKK (phosphatidylinositol-3-kinase-like kinase) family: ATM (ataxia-telangiectasia mutated), ATR (ataxia-telangiectasia and Rad3-related), and DNA-PK (DNA-dependent protein kinase). While all three are required for proper genomic maintenance, DNA-PK lacks the capacity to elicit many of the effects induced by ATM or ATR. For this reason, DNA damage signaling (DDS) generally is considered to occur mainly through ATM and ATR. Recent studies, however, have implicated that DNA-PK can regulate DDS through hindrance of ATM-DDS, giving rise to an evolving view in which all three PIKK family members are essential for regulation of DDS, but not its initiation. This chapter presents a discussion of the signaling within human systems induced by DNA damage as well as an overview of the roles of DDS in promoting DDR-mediated cell cycle arrest, DNA damage repair, and changes to other cellular processes. Within this context, the roles of DDR in current and proposed chemotherapeutics will be explored.

Keywords DNA Damage • DNA Damage Response • Cell Cycle Regulation • Cancer • Chemotherapeutics

B.M. Cartwright • P.R. Musich • Y. Zou (✉)
Department of Biomedical Sciences, Quillen College of Medicine,
East Tennessee State University, Johnson City, TN, USA
e-mail: zouy@etsu.edu

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N. Shinomiya et al. (eds.), *Regulation of Signal Transduction in Human Cell Research*, Current Human Cell Research and Applications,
https://doi.org/10.1007/978-981-10-7296-3_6

6.1 Introduction

Every day cells are faced with a barrage of genomic insults from endogenous and exogenous sources. Inevitably, these insults lead to DNA damage which must be repaired to maintain genomic integrity. Cells respond to DNA damage by initiating various signaling events. This marks the beginning of the DNA damage response (DDR), and these events lead to activation of cellular pathways ranging from cell cycle arrest, DNA repair, and even cell death in cases of excessive damage. Because of these diverse signaling events, the DDR can regulate cellular fate to promote genomic fidelity on multiple levels. The importance of the DDR is further highlighted by the fact that individuals deficient in DDR function are typically cancer-prone as well as exhibit a wide variety of other pathological complications [1–4].

The DDR is primarily initiated by three PIKK (phosphatidylinositol-3-kinase-like kinase) family members: ATM (ataxia-telangiectasia mutated), ATR (ataxia-telangiectasia and Rad3-related), and DNA-PK (DNA-dependent protein kinase). ATM, ATR, and DNA-PK are serine/threonine kinases that maintain genomic integrity through direct regulation of DDR. While all three kinases are required for proper genomic maintenance, DNA-PK is typically seen as dispensable for overall DNA damage signaling (DDS) [3]. Recently, however, studies have challenged this notion with findings that DNA-PK can regulate ATM activity through direct phosphorylation of ATM as well as potentially mediate cell cycle arrest through Aurora B [5, 6].

In the following sections, the roles of ATM, ATR, and DNA-PK in response to DNA damage will be addressed. First will be a discussion of the roles of the major DDR pathways and PIKK family members in regulation of cell cycle arrest and DNA repair. Then, after briefly reviewing other DNA damage-induced responses, the potential role of chemotherapeutics in eliciting or modulating DDS and the DDR will be considered.

6.2 Maintaining Genomic Fidelity: Cell Cycle Control and DNA Repair

After cells experience DNA damage, there is a complex interplay of signaling pathways. These pathways, mainly orchestrated through ATM and ATR, lead to a fine-tuned response which alters cell cycle progression, nucleotide metabolism, and other parameters involved in promoting an optimal environment for maintaining DNA integrity [2, 4]. The following sections address cell cycle checkpoint activation and control, various types of DNA repair, and the multifaceted interplay through which checkpoint activation modulates the DDR.

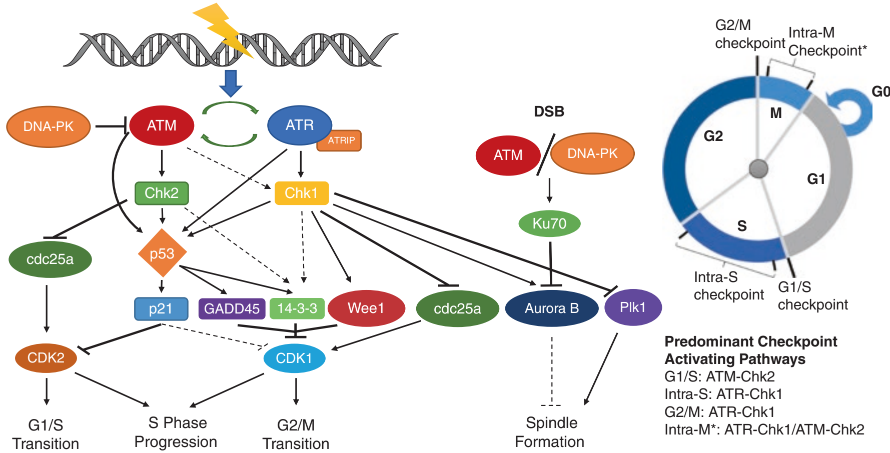

Fig. 6.1 Cell cycle regulation by the DNA damage checkpoints. DDS through ATM, ATR, and DNA-PK control the cell cycle at multiple levels. ATM and ATR are the primary regulators of cell cycle arrest through their downstream substrates, kinases Chk2 and Chk1 respectfully. Chk1 and Chk2 phosphorylate multiple targets resulting in either their direct inhibition, degradation, or activation of negative regulatory function. DNA-PK is thought to play a lesser role in DDS, though it has been shown to regulate ATM activity as well as potentially be involved in spindle formation checkpoint (*intra-M checkpoint). DNA repair pathway engagement is cell cycle-dependent. Where BER, NER, and NHEJ occur in all phases of the cell cycle, HR and ICLR only occur during S and G2 phases

6.2.1 Cell Cycle: Checkpoint Control and Arrest

When cycling cells incur DNA damage, they activate processes to arrest cell cycle progression. This arrest prevents the accumulation of mutations in both themselves as well as potential daughter cells. While the exact regulation of cell cycle progression is out of the scope of this review, it is important to note that DDS mediated by both ATM and ATR regulate the cell cycle through downstream modulation of cell cycle progression factors (Fig. 6.1).

ATM and ATR function to regulate the cell cycle in response to different stresses and largely during different phases of the cell cycle. ATM and its effector kinase Chk2 (checkpoint kinase 2) respond mainly to DNA double-strand breaks (DSBs). While ATM is active throughout the cell cycle, it plays a predominant role during the G1 phase of the cycle and into the G1/S transition. During this phase, the cells are preparing to synthesize new DNA, and damage occurring during G1 leads to activation of ATM through autophosphorylation on its Ser1981 residue which leads to the dissociation of the ATM dimer to monomer and further phosphorylation of ATM at Ser367, Ser1983, and Ser2996 [7, 8]. ATM then phosphorylates Chk2 on Thr68, leading to its activation and priming the cell cycle arrest cascade [3, 9]. Chk2 has a myriad of substrates; however, two are of primary interest regarding cell cycle arrest: cdc25A and p53. Chk2 phosphorylates the protein phosphatase cdc25A on Ser123, limiting its activity as well as targeting it for ubiquitination and subsequent

proteasomal degradation. Restriction of cdc25A activity prevents the removal of the inhibitory phosphoryl groups attached to Thr14 and Tyr15 of CDK2 (cyclin-dependent kinase 2). CDK2, when complexed with cyclin E, is required for the progression from the G1 to the S phase of the cell cycle. Therefore, reduction of cdc25A activity facilitates arrest of the G1/S transition due to decreased activation of CDK2 [9, 10]. In addition to down regulation of CDK2 through inactivation and removal of cdc25A, ATM and Chk2 coordinate the induction of p21$^{WAF1/Cip1}$ which directly inhibits CDK2 through interaction via its N-terminal Cy1 motif [11]. This induction is part of a three-step mechanism. The first step is the phosphorylation of p53 on Ser20 by Chk2, leading to a conformational shift in p53 allowing for its dissociation from its normal sequestering protein MDM2 (mouse double-minute 2 homolog), an E3 ubiquitin ligase. This dissociation allows the second step to occur in which p53 is further phosphorylated by ATM at Ser15. Modification of this site increases the transcriptional activity of p53 resulting in the induction of p21$^{WAF1/Cip1}$. The third step involves ATM phosphorylation of MDM2 on Ser395, serving as a backup mechanism to prevent its rebinding to p53 and, thus, ensuring proper p53 activation and induction of increased transcription of DDR protein genes [9, 12].

While the G1/S checkpoint is initiated through ATM, ATR and its effector kinase Chk1 (checkpoint kinase 1) are the primary activators of the intra-S (primarily in response to DNA damage-induced replicative stresses) and G2/M checkpoints. Longer single-strand DNA (ssDNA) generated through replicative stress or DSB end resection is rapidly coated with replication protein A (RPA), the RPA-ssDNA complex recruits ATR-ATRIP (ATR-interacting protein) as well as Rad17 and the 9–1-1 (Rad9-Rad1-Hus1) complex. Binding of the 9–1-1 complex to RPA is a signal for TopBP1 (topoisomerase-binding protein 1) recruitment [13–16]. TopBP1 is an allosteric regulator of ATR, promoting ATR activation through its autophosphorylation on Thr1989 [17–19]. After complexing with claspin, activated ATR then phosphorylates Chk1 on Ser317 and Ser345 to elicit intra-S and G2/M checkpoint activation and arrest of cell cycle progression [20]. Like Chk2, Chk1 also phosphorylates cdc25A on Ser123, leading to its inhibition and targeted ubiquitin-mediated degradation. While this action promotes the G1/S checkpoint, it also serves as a safeguard should cells be able to complete DNA repair and reenter the cell cycle. Additionally, this functions to facilitate the intra-S checkpoint through the inability of phosphorylated CDK2 to form an active CDK2/cyclin A complex, resulting in premature stalling or termination of DNA synthesis [20, 21]. The intra-S checkpoint is mediated further by parallel phosphorylation of p53 by ATR and the subsequent gene induction cascade as presented previously through p53 with respect to ATM and Chk2.

In addition to p21$^{WAF1/Cip1}$ induction, p53 also promotes the transcriptional upregulation of GADD45 and 14–3-3 which both regulate the G2/M checkpoint upon ATM and ATR activation. GADD45, commonly known as GADD45a, functions to directly bind to and suppress CDK1 (cyclin-dependent kinase 1) while it is in complex with cyclin B1. In this way, binding of GADD45 to the CDK1/cyclin B1 complex inhibits CDK1 activity and the transition from G2 into mitosis. Of importance also is the fact that GADD45 has no effect on the activity of the CDK1/cyclin E complex which is active during G1 further tailoring GADD45 as a G2/M checkpoint inducer [22]. The

active forms of both Chk1and Chk2 phosphorylate cdc25C at Ser216 priming it for binding by the 14–3-3 complex which leads to the nuclear export and cytosolic sequestration of cdc25C. This sequestration is necessary for G2/M checkpoint arrest because cdc25C, a protein phosphatase similar to cdc25A, functions to remove inhibitory phosphorylations from CDK1. Unphosphorylated CDK1, complexed with cyclin B1, serves as the regulatory kinase for cell cycle progression from G2 into mitosis. Therefore, like with CDK2, inhibition of the activating phosphatase leads to an increased accumulation of inactive phosphorylated CDK1 [23]. These mechanisms, however, are not alone in the regulation of the G2/M checkpoint; Chk1 also activates Wee1, a kinase that phosphorylates CDK1 on Thr14 and Tyr15. This phosphorylation inhibits CDK1 activity. These phosphorylations by Wee1 enhance the inhibition of CDK1 brought about by the cytoplasmic sequestration of the protein phosphatase cdc25C [24]. To further promote cell cycle arrest, downstream kinases activated by ATM and ATR lead to phosphorylation of Plk1 (Polo kinase 1), targeting it for degradation. The degradation of Plk1 promotes a prolonged and robust G2/M arrest by Wee1 as under normal physiological conditions Plk1 phosphorylates Wee1, leading to its degradation and allowing for a normal G2/M transition [25].

Lastly, the intra-M, or mitotic spindle checkpoint, serves as a last line of defense in protecting genomic integrity following DNA damage. Unlike the other checkpoints, all three DDR PIKK family members are involved in the initiation of the intra-M checkpoint. ATR functions through Chk1 to activate the Aurora B kinase which subsequently delays abscission and progression through cytokinesis [26]. Additionally, Chk1 phosphorylates Plk1 preventing its active role in promoting centrosome formation and mitotic spindle assembly [25]. ATM and DNA-PK are implicated in the intra-M checkpoint via regulation of Ku70 phosphorylation at Ser155 [5]. While it is unknown if this phosphorylation is dependent on ATM or DNA-PK, this phosphorylation event is of importance as it leads to the interaction of Ku70 with Aurora B, inhibiting the latter's kinase activity [5].

It is important to note that activation of ATR and ATM are both likely to invoke the DDR and cell cycle arrest regardless of cell cycle phase due to known cross activation and regulation between the DDS pathways [27–30]. In addition, recent reports have shown that DNA-PK is capable of directly modulating ATM activation through inhibitory phosphorylation of ATM, leading to reduced DDS through ATM following DNA damage. Because of this, DNA-PK could potentially regulate ATM induction of checkpoints and subsequent cell cycle arrest [6]. This implicates an even more complex regulation of DDR than previously described.

6.2.2 DNA Damage Repair

Upon sensing of DNA damage, cells activate repair processes to restore genomic integrity. Multiple DNA repair mechanisms have evolved to defend genomic integrity against a variety of different endogenous and exogenous sources of DNA damage (Fig. 6.2). Specific repair pathways engage depending on the type of lesions

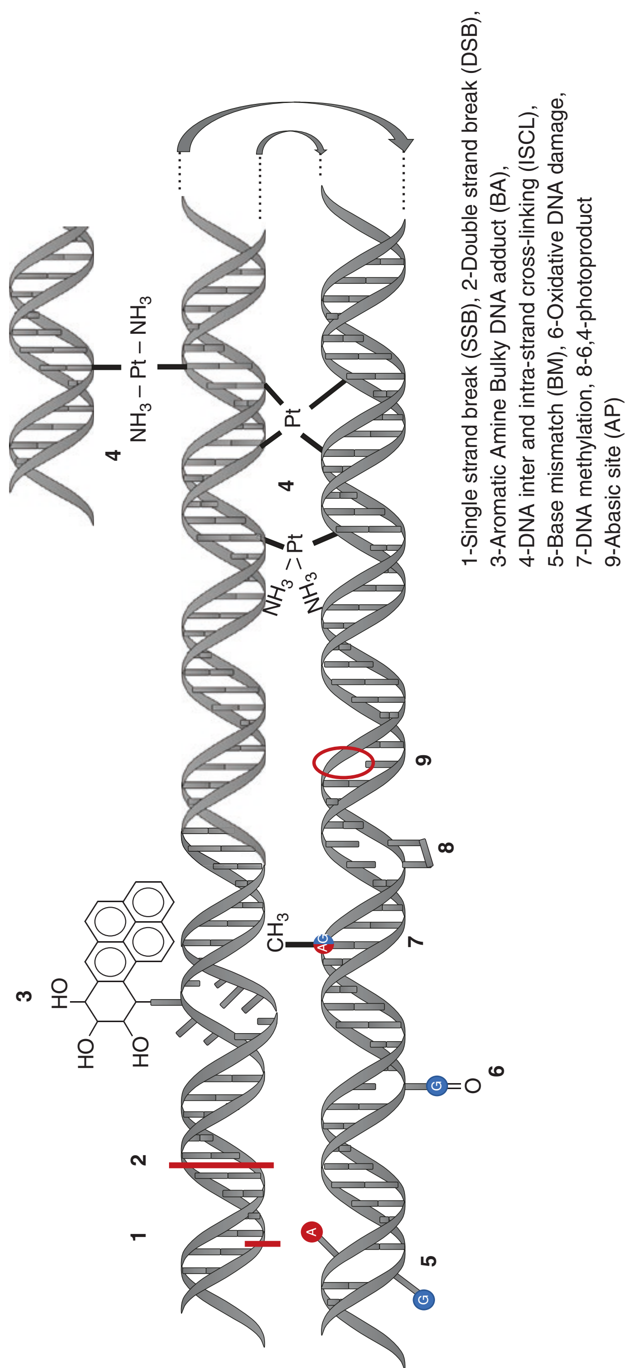

Fig. 6.2 Common types of DNA damage

Table 6.1 Types of DNA repair and activating factors

DNA repair pathway	Types of damage
BER	Alkylation, spontaneous depurination or depyrimidination, deamination, oxidation, single-strand breaks
NER	Bulky adducts (benzo(*a*)pyrene, photoproducts, etc.), intrastrand cross-links
MMR	A > G mismatches, T > C mismatches, trinucleotide expansions, base deletions
NHEJ/Alt-NHEJ	Double-strand breaks
HR	Double-strand breaks
ICLR	Inter- and intrastrand cross-links

present (Table 6.1). The following is a summary of major pathways as well as the DDS effects that influence them.

6.2.2.1 Base Excision Repair (BER)

The most commonly occurring lesions in DNA are those arising from oxidation, alkylation, or spontaneous depurination/depyrimidination (abasic site formation). These lesions trigger the base excision repair (BER) process facilitating the removal of the damaged base and processing of the newly generated abasic site (Fig. 6.3a). The initial step in BER is hydrolysis of the N-glycosylic bond of the modified base by various DNA glycosylases leading to the formation of an apurinic/apyrimidinic (AP) site. The AP site then is modified by poly (ADP-ribose) polymerase 1 (PARP1) through a process known as PARylation. PARylation is a process in which a polymer of ADP-ribose (PAR) is added to DNA or proteins through consumption of NAD^+ [1, 31]. This PARylation event generates the primary signal of DDS in BER as PARylation of DNA, as well as auto-PARylation of PARP1, leads to the recruitment of several downstream proteins associated with BER including XRCC1 (X-ray repair cross-complementing protein 1), OGG1 (8-oxoguanine glycosylase 1), and others. This recruitment is based on binding to PAR chains facilitated through various binding motifs [31].

In addition, previous reports have shown that protein deacetylases can influence DDS signaling of BER through the initial substrate specificity of glycosylases [32]. Examples include the effects of SIRT1 on TDG (thymine DNA glycosylase) and APE1 (AP-endonuclease 1). SIRT1 deacetylation of TDG changes the substrate specificity of TDG, whereas deacetylation of APE1 promotes binding with XRCC1. In the case of TDG, the deacetylation by SIRT1 promotes excision of the nucleoside analogue 5-fluorouracil (5-FU), whereas unacetylated TDG mainly targets methylated substrates [33, 34]. Loss of SIRT1 deacetylation has the potential of muting the DDR by preventing the recognition of the lesion and the generation of the AP site. The same is true of APE1 whose deacetylation at Lys6 and Lys7 by SIRT1 leads to interaction with XRCC1. This

APE1-XRCC1 interaction promotes the glycosylase activity of APE1, enhancing AP site generation [35].

Once PARP1 modifies the AP site, other repair proteins are recruited to finish processing the AP site. WRN (RecQ or Werner protein), a helicase and exonuclease, is recruited to stimulate polymerase β (POLβ) binding for insertion of the missing nucleotide or nucleotides. DNA ligase III (LIGIII) then ligates the DNA strand [36]. This process is DDS-independent; however, it has been shown that DDS-dependent deacetylation of WRN by SIRT1 promotes its exonuclease activity in cases of long-patch BER. In this way, signaling by SIRT1 functions to improve BER endonuclease activity which leads to the removal of up to ten nucleotides [37]. Under long-patch BER, polymerase δ (POLδ) or polymerase ε (POLε) catalyzes the repair DNA synthesis; FEN1 (flap endonuclease I) then removes the displaced DNA strand. The DNA strands are then ligated by DNA ligase I (LIGI) [32].

6.2.2.2 Nucleotide Excision Repair (NER)

Bulky DNA lesions, such as those caused by UV-induced photoproducts, polycyclic aromatic hydrocarbons, and cross-linking chemotherapeutics (e.g., *cisplatin*), can distort the DNA helical structure. Nucleotide excision repair (NER) repairs these types of lesions (Fig. 6.3b). NER pathways come in two distinct forms: global genome repair (GG-NER) and transcription-coupled repair (TC-NER) [38–40].

In human GG-NER, initial recognition of DNA damage is done by XPC (XP complementation group C)-HR23B or together with DDB1-DDB2/XPE [damage-specific DNA-binding protein 1 or 2- xeroderma pigmentosum complementation group E (XPE)] upon UV irradiation. While the XPC-HR23B complex can localize to damaged DNA by itself, the efficiency of recruitment is enhanced following polyubiquitination by the DDB1-DDB2-CUL4A/B complex. This polyubiquitination assists XPC in binding at the DNA lesion [41]. Once XPC binds to a bulky DNA adduct, transcription factor IIH (TFIIH) is recruited. Two components of the TFIIH, XPB and XPD (XP complementation groups B and D, respectively), mediate strand unwinding. The single-strand DNA (ssDNA) generated is rapidly bound by RPA (replication protein A), and XPA (XP complementation group A) is recruited for verification of the DNA damage. XPA also appears to stabilize the repair intermediate and serves to recruit the endonucleases XPG (XP complementation group G) and XPF-ERCC1 (excision repair cross-complementation group 1) [42, 43]. XPG facilitates the 3′ incision, while XPF-ERCC1 does the 5′ incision [44, 45]. Then, the adducted DNA fragment of 22–30 nucleotides is removed, followed by recruitment of PCNA (proliferating cell nuclear antigen) by RFC (replication factor C). PCNA loads one of three DNA polymerases (delta, epsilon, or kappa) onto the DNA facilitating its repair synthesis. DNA ligase I or the ligase III-XRCC1 complex then seals the DNA termini [38]. TC-NER follows a similar series of steps with the exception that ERCC6/CSB (excision repair cross-complementing group 6, Cockayne syndrome B) performs the initial damage recognition, not XPC-

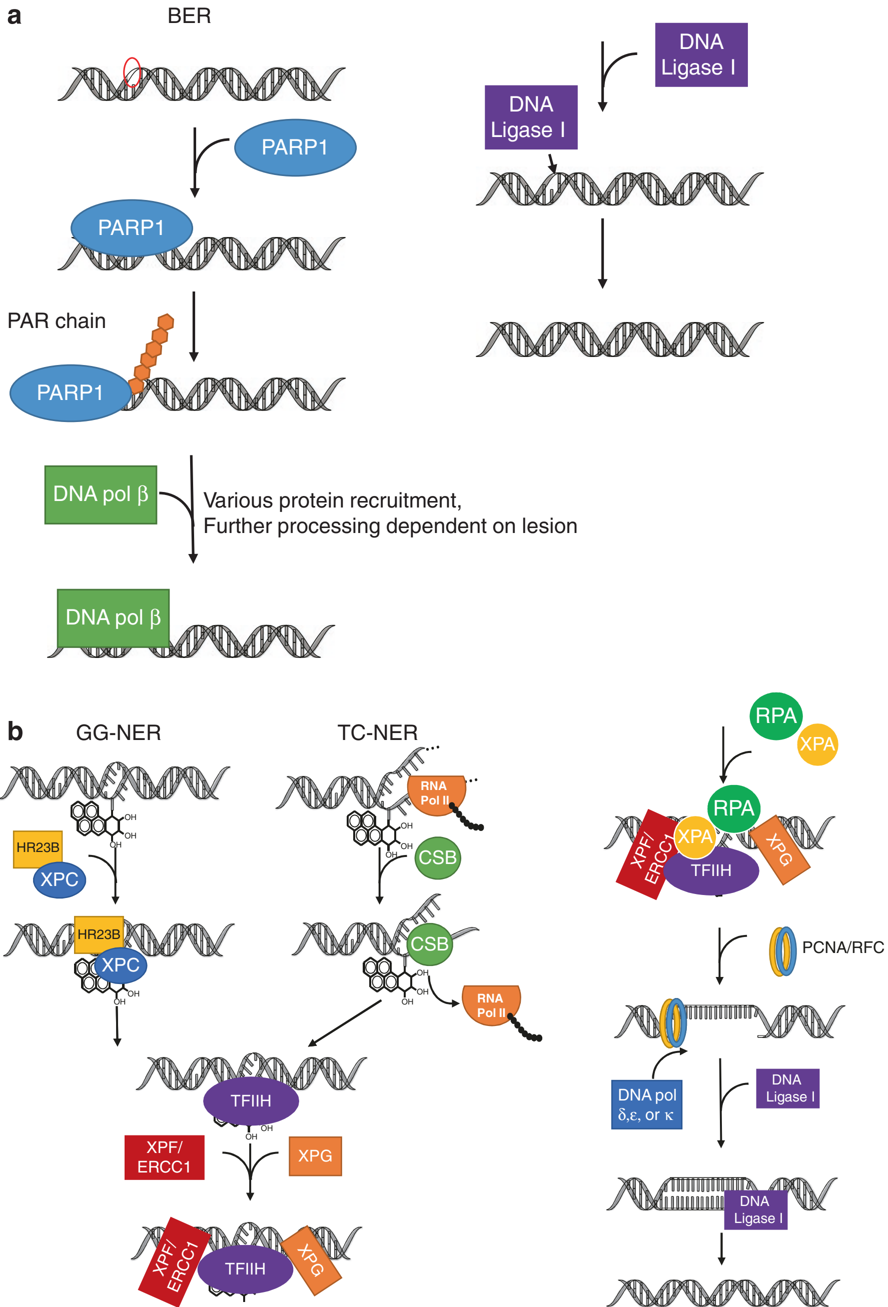

Fig. 6.3 Selected DNA damage repair pathways. DNA damage must be repaired to prevent mutations from occurring within cells. (**a**–**d**) summarize some of the most common forms of DNA damage repair. (**a**) Base excision repair (BER) [short patch of an apurinic site], (**b**) nucleotide excision repair (NER) of a bulky aromatic adduct, (**c**) nonhomologous end joining (c-NHEJ and alt-NHEJ), (**d**) homologous recombination (HR)

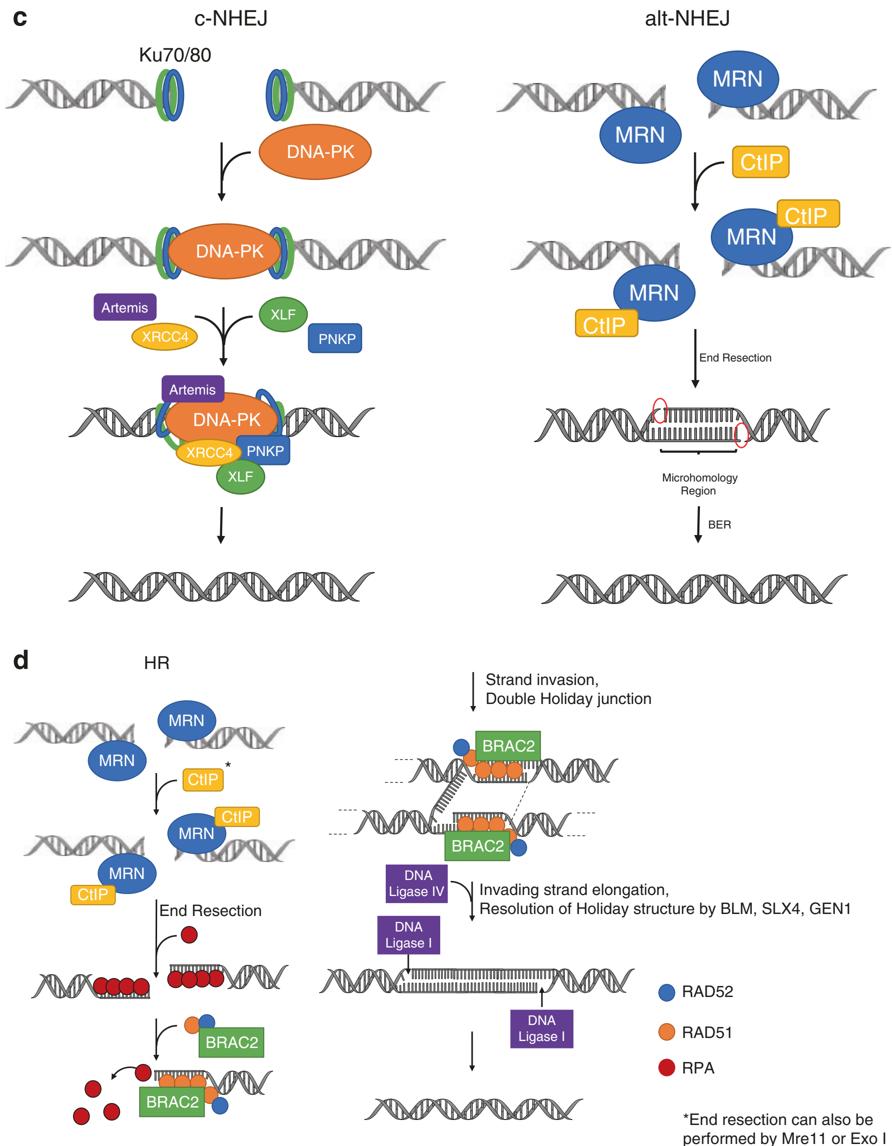

Fig. 6.3 (continued)

HR23B, and is followed by the removal of stalled RNA polymerase II from the DNA lesion before repair proceeds. This is done through ubiquitin-mediated removal and subsequent degradation that is dependent on ERCC6/CSB [46].

In addition, some other key events are involved in NER. For example, SIRT1 deacetylation of XPA promotes the interaction of XPA with other NER factors. This serves to increase recruitment and activity of XPG and ERCC1-XPF [47].

Additionally, ATR-mediated XPA phosphorylation enhances XPA stability by inhibiting HERC2-medated ubiquitination and subsequent degradation [48].

6.2.2.3 Mismatch Repair (MMR)

Mismatching of bases typically occurs during replication or after the deamination of cytosine in DNA. In brief, either MutSα (MSH2-MSH6 heterodimer) or MutSβ (MSH2-MSH3 heterodimer) binds to the mispaired bases. MutSα preferentially recognizes post-replicative mispairings as well as methylated bases, whereas MutSβ recognizes insertion repeats or deletion loops [49, 50]. Both versions of MutS then serve as a scaffold signaling the recruitment of various factors to excise and replace the mismatched or damaged DNA. Active HDAC 6 and 10 decrease the stability of MSH2 and enhance its degradation, indicating that acetylation of MSH2 is required for scaffold stability and MMR [32].

6.2.2.4 Nonhomologous End Joining (NHEJ)

DSBs are repaired by two major pathways, the nonhomologous end joining (NHEJ) and homologous recombination (HR). NHEJ corrects strands brakeage through direct modification and ligation of broken strands without regard for sequence homology. To date, two distinct forms of NHEJ have been discovered: classic-NHEJ (c-NHEJ) and alternative-NHEJ (alt-NHEJ) (Fig. 6.3c) [51].

c-NHEJ initiates upon binding of Ku70/Ku80 heterodimer to the termini of DSBs. This leads to the recruitment of DNA-PKs to the Ku70/Ku80-bound DNA. Upon DNA binding, DNA-PK undergoes activation and autophosphorylation at multiple sites in both its ABCDE (T2609, S2612, T2620, S2624, T2638, and T2647) and PQR (S2023, S2029, S2041, S2051, S2053, and S2056) domains [52]. These events promote stability of the DNA-PK/DNA complex and facilitate end interaction while recruiting other factors such as Artemis, XRCC4, XLF (XRCC4-like factor), PNKP (polynucleotide kinase/phosphatase), and DNA ligase IV [53]. If the broken DNA ends are not compatible for direct ligation by DNA ligase IV, they are first processed. The Artemis nuclease is responsible for end resection during this process. PNKP then modifies the ends of the DNA to be recognizable substrates for DNA ligase IV by either removing or adding phosphoryl groups from the 3′ and 5′ termini, respectively. While DNA-PK phosphorylates all of the proteins it recruits, currently there is no evidence that any of these individual phosphorylation events are necessary for NHEJ [54].

An early event following DNA-PK activation is the phosphorylation of histone variant H2AX at Ser139; this phosphorylated form is known as γH2AX. This is an important step as γH2AX serves to amplify the signal of the DSB and aids in the recruitment of many factors involved in repairing the breakage by promoting chromatin reorganization. One of the factors recruited is ATM which modulates NHEJ to either promote its efficiency or to promote a shift to HR [30, 55]. The molecular

processes governing pathway decision for DSB repair are presented in the homologous recombination (HR) section (Sect. 2.2.5).

There are many factors which modulate c-NHEJ efficiency. PARylation by PARP1 directly stimulates DNA-PK activity. The PARP1 binding to the DNA-PK/Ku complex elicits a structural change in the complex, facilitating more efficient repair [31]. PARylation of DSB termini also increases early recruitment of DNA ligase IV through a scaffolding event caused by interaction between the BRCT domain of DNA ligase IV and the PAR chains. This has the potential to promote a more efficient repair due to decreased lag time in recruitment of DNA ligase IV [56]. Deacetylation also plays a distinct role in NHEJ. KAP1 (KRAB-associated protein-1) is deacetylated by SIRT1 which promotes chromatin relaxation and invasion of NHEJ repair factors. SIRT1, as well as HDAC 1–3, deacetylates Ku70, promoting its binding to DSB termini, and subsequently increases NHEJ efficiency. Lastly, SIRT6 promotes DNA-PK localization to DSBs as well as DNA-PK/Ku complex stability [32].

Alt-NHEJ is independent of both DNA-PK and Ku70/80. In alt-NHEJ, PARP1 recognizes DSBs which have already undergone end resection and have complementary microhomology regions (1–10 nucleotides) which have annealed. PARP1 then PARylates the termini of the breaks, signaling for DNA ligase III/XRCC1 recruitment and ligation of what now appear as SSBs. Alt-NHEJ is more error prone than c-NHEJ as it requires formation of microhomologies and greater amounts of DNA may be resected prior to annealing to facilitate microhomology formation [31, 57].

6.2.2.5 Homologous Recombination (HR)

Homologous recombination is imperative for the maintenance of genomic stability during development and preservation of stem cell populations. Active in S and G2 phases of the cell cycle, HR (Fig. 6.3d) requires both ATM and ATR kinases to function.

In HR, the MRN complex, made up of Mre11, Rad50, and Nbs1, recognizes DSBs leading to the recruitment of ATM. Once bound to the MRN complex, ATM undergoes activation through autophosphorylation at Ser1981. Another early ATM substrate in this process is histone H2AX which is rapidly phosphorylated at Ser139 to form γH2AX, which induces recruitment of MDC1. MDC1 serves to form an adaptor complex with ATM-Nbs1 as well as with γH2AX. The MDC1-ATM-Nbs1 complex amplifies the γH2AX signal through further phosphorylation of H2AX, while the MDC1-ATM-γH2AX complex recruits E3 ubiquitin ligase RNF8. RNF8 ubiquitinylates various histones surrounding the DSB site, serving to loosen the local chromatin structure as well as provide a signal for recruitment RNF168, another E3 ubiquitin ligase, through its ubiquitin-binding domain. RNF8 and RNF168 function through the E2 ubiquitin ligase UBC13 to promote the recruitment and retention of various NHEJ and HR factors: 53BP1, RAD18, BRCA1, BRCA1-A, HERK2, etc. [55, 58].

For homologous recombination to continue, the DSB ends must have one strand resected. One of three exonucleases performs this: Mre11, Exo1, or CtIP [59–61]. The BRAC1/BARD1 complex enhances this resection and facilitates pathway selection through direct displacement of 53BP1 from DSBs as well as through recruitment of various factors required for the end resection through its BRCT motifs. BRAC1 and 53BP1 are both recruited through RNF8 signaling; however, they function in a dynamically opposed manner. While BRAC1 promotes end resection and repair through HR, 53BP1 functions to inhibit end resection and promote repair through NHEJ [58]. So why would BRAC1 and 53BP1 both be recruited by the same initial signaling event? This can be attributed to 53BP1 being necessary for effective ATM activation as well as being required for ATM-mediated checkpoint kinase activity through Chk2 [62]. In this way, both BRAC1 and 53BP1 are required for appropriate HR function if only to allow more time to complete the required repair.

The BRAC1/BARD1 complex promotes the recruitment of Abraxas-RAP80, BRIP1 helicase, and CtIP. BRAC1 forms a complex with each protein partner to form either BRAC1-A, BRAC1-B, or BRAC1-C, respectively. BRAC1-C functions to induce end resection through CtIP's exonuclease activity. The BRAC1-A complex regulates this resection to ensure that ends are not over processed. BRAC1-B removes secondary DNA structures that occur during this process as well as alleviating any occurring before end resection that might be due to cross-linking, replication fork stalls, or replication fork collapses [63, 64].

The ssDNA generated by end resection is rapidly bound by RPA. Following RPA binding, recombination initiates with the Rad52-mediated loading of Rad51 recombinase onto the ssDNA of resected DSB. Rad51 displaces RPA from ssDNA and is dependent on the BRAC2-PALB2 complex which functions to localize BRAC2 and Rad51 to the ssDNA and allows for efficient Rad51 loading by BRAC2 [64, 65]. Rad51 plus other HR proteins forms the nucleoprotein filament that is responsible for sister chromatid invasion. During this process, Rad51 mediates strand displacement and invasion of the 3′ end of that strand into the sister chromatid, resulting in the formation of a D-loop structure with the invading strand base-paired to the intact complementary strand. DNA synthesis then extends the invading strand resulting in Holliday junction formation. Once this junction is formed, the other 3′ strand of the DSB enters the complex leading to formation of a double Holliday junction where this strand then is elongated. The resolution of these structures is still not well understood in eukaryotic systems, but several proteins such as BLM, MUSLX4, or GEN1 could be implicated due to their helicase and nuclease activities [66–68].

In addition to the responses presented above, there are other measures which help to ensure pathway selection and successful completion of HR. One example of this is deacetylation of CtIP by SIRT6 which promotes its function in end resection. SIRT6 depletion decreases the amount of RPA recruited to resected DSBs implicating its importance in supporting HR [69]. This contrasts with its function in supporting NHEJ, as presented previously, and likely has cell cycle dependence, as HR is only available during S and G2 phases.

6.2.2.6 Interstrand Crosslink Repair (ICLR)

Some lesions are highly complex and require multiple pathways to repair them efficiently. The repair of interstrand cross-links requires activation of NER and/or HR in combination with the Fanconi anemia (FA) pathway. The combined process, known as interstrand cross-link repair (ICLR) for simplicity, is initiated by either XPC or ERCC6/CSB if the cross-link can be removed through NER or by the FA pathway protein FANCM (Fanconi anemia complementation group M) if it is within a stalled replication fork [41, 46, 70]. As NER was previously described (Sect. 2.2), the following will address the role of FA in ICLR.

Initial recognition of an interstrand cross-link at a stalled replication fork is performed by FANCM which is part of the Fanconi anemia core complex: FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, and FANCM. FANCM along with binding partners FAAP24 and MHF1 forms a complex that supports the DEAH-helicase activity of FANCM leading to strand displacement at the branches of a stalled replication fork [71]. This promotes the accumulation of RPA on the displaced ssDNA and subsequent likely recruitment and activation of ATR/ATRIP as is seen in HR [72]. Simultaneously, FANCL induces ubiquitination on FANCD2 a Lys561. This is crucial for FANCD2/FANCI complex localization to the site of DNA damage [73]. FANCD2/FANCI promotes the recruitment of FAN1 and SLX4 (FANCP) which function alongside XPF/ERCC4 (FANCQ) to perform incisions upstream and downstream of the interstrand cross-link, respectively [74]. After the incisions, HR takes over the DNA repair process. It is important to note that when the FA pathway is referenced, there are several alternative names for HR proteins, but otherwise they possess the same function. These are as follows: BRAC2 (FANCD1), PALB2 (FANCN), BRIP1 (FANCJ), and RAD51 (FANCO).

ATR serves a particular role in the FA pathway because its DDS activity is required to ensure ICLR through the main FA complex as well as the FANCD2/FANCI complex. First is the ATR-dependent phosphorylation of FANCM at Ser1025 which is required for FA pathway activity and sufficient G2/M arrest [75]. ATR also activates the FANCD2/FANCI complex through phosphorylation of FANCD2 at Thr691 and Ser717 and FANCI at several SQ/TQ motifs [76, 77]. ATR also phosphorylates FANCA at Ser1449 and promotes phosphorylation of FANCE by Chk1 [78, 79].The result of which in all instances promotes FANCD2 ubiquitination, recruitment to DNA damage sites, and activity [74].

6.2.3 Integrating the Signals: Checkpoint Regulation of DNA Repair

When considering the DDR, it is essential to consider the transiently activated checkpoints as well as its regulation of DNA repair as coordination between the two systems is indispensable for successful maintenance of genomes. Also importantly, the coordination is likely cell cycle-dependent in most cases. While this is based on

the premise that some DNA repair protein expression is limited to certain phases of the cell cycle, this concept has much broader implications when investigating integrated control on pathway selection and repair efficiency [80–82].

The checkpoint control of DNA repair is seen at multiple levels stemming from ATM, ATR, and DNA-PK activity. For instance, ATR regulates NER following UV irradiation through direct binding and phosphorylation of XPA at Ser196 promoting its stability and nuclear import following UV irradiation [83–86]. This process is dependent on PKA phosphorylation of ATR at Ser435, and loss of this site leads to reduced ATR-XPA binding as well as delayed XPA recruitment to sites of DNA damage [87]. This effect is found to occur primarily in the S phase of the cell cycle and to be p53 dependent. This is in contrast to XPA nuclear import during G1 or G2 phases in which XPA nuclear import is p53/ATR independent; in G1, the UV-induced import is muted, while in G2, XPA accumulates in the nucleus regardless of DNA damage [88]. Checkpoint control of NER is further enacted by p53 which is a target of all three apical kinases as well as secondary kinases, Chk1 and Chk2. p53 upregulates gene expression of NER proteins following a variety of genomic insults resulting in increased DDB2, XPC, XPF, and XPG levels [89, 90]. Active p53 is also known to be involved in the recruitment of XPC as well as TFIIH to sites of UV damage where it facilitates improved DNA damage recognition and repair [91, 92].

Checkpoint control of DNA repair also extends to BER, HR, and NHEJ. BER activity is modulated through activated p53's direct binding to three BER enzymes: APE/REF1, OGG1, and DNA polymerase beta. This binding stimulates the recognition, excision, and respective repair activities of these enzymes leading to enhanced BER [93, 94]. ATM, ATR, and DNA-PK all collaborate to promote effective HR through the regulation of the RPA-p53 interaction [30]. The interaction of RPA-p53 typically promotes NHEJ through sequestration of RPA; however, Ser37 and Ser46 phosphorylation of p53 by ATM and ATR, respectively, along with RPA32 phosphorylation by DNA-PK leads to dissociation of the RPA-p53 complex and a switching to HR [30, 95].

In addition to the effects listed previously, direct cycle control of DNA repair occurs through cyclic expression of repair factors and regulation by cyclin dependent kinases. In brief, many proteins involved in DNA repair are only expressed at certain points throughout the cell cycle. For instance, gene-encoding proteins for mismatch repair are almost exclusively expressed in S phase, whereas most genes for ICLR are expressed in S-M phases. For more information regarding cell cycle expression of DNA repair proteins, we would point interested readers to the recent work by Mjelle et al. as the topic is quite expansive [80]. Cyclin-dependent kinases also play a role in promoting DNA repair efficiency and pathway selection. CDK1, the cyclin associated with promoting G2/M transition, is responsible for phosphorylation of CtIP at Ser327 and Thr847 leading to increased BRCA1 binding by CtIP and enhanced end resection favoring HR repair [96, 97]. In contrast, CDK2, which is responsible for promoting the G1/S transition, phosphorylates CtIP at Ser276 and Thr315 leading to its inhibitory isomerization by prolyl isomerase Pin1. This prevents end resection of DSBs in early G1 and early S phases leading to the promotion of NHEJ over HR in these instances [98]. These events represent a minor fraction of the cell cycle control of DNA repair. For more information, we would suggest the recent works by Hustedt and Warmerdam [81, 82].

6.3 Other Cellular Responses to DNA Damage

Cells respond to DNA damage in an assortment of ways in addition to the DDR. Most of these responses are geared at increasing DNA repair efficiency and promoting cell survival. Processes such as autophagy and inflammation serve to facilitate these pro-survival responses; in contrast, there are times where the DNA damage is too extreme to allow for cell survival. While cell death may be viewed as a negative event, under normal conditions it serves to protect the organism from tumorigenesis. In cases of extreme DNA damage, ATM and ATR can initiate pro-apoptotic signaling through the tumor suppressor p53. These responses, and their interplay with processes previously mentioned, are detailed in the following sections.

6.3.1 Autophagy

Autophagy is a catabolic process by which proteins and organelles are degraded to either remove damage or provide usable metabolic constituents in times of stress. Typically, autophagy is induced under cellular damage or starvation; however, it also can be induced by other taxing events such as DNA damage [99]. Several studies have established that the induction of autophagy following DNA damage is cytoprotective and plays an integral role in protecting cells upon DNA damage induced by chemotherapy, radiation, or other sources [100–102].

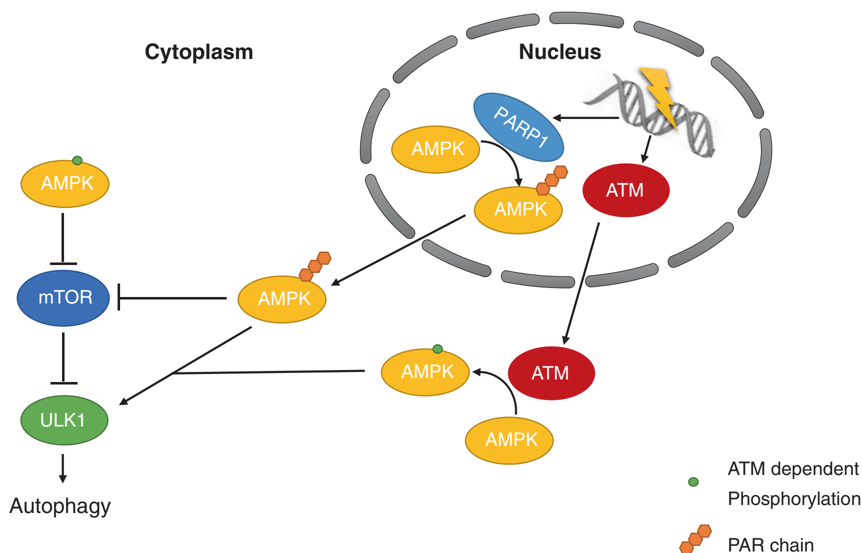

Fig. 6.4 DNA damage regulation of autophagy

The regulation of autophagy by the DDR occurs on multiple levels (Fig. 6.4). The regulation of autophagy by the DDR can occur through ATM which phosphorylates cytoplasmic AMPK (5′ adenosine-monophosphate-activated protein kinase) on Thr172 resulting in its activation. This leads to the induction of autophagy by two mechanisms. The first is inhibition of the kinase mTOR (mammalian target of rapamycin) through activation of TSC1/2 (tuberous sclerosis complex 1 or 2). AMPK phosphorylation of TSC1/2 causes TSC1/2 interaction with mTOR, leading to repression of mTOR activity. Under normal conditions, mTOR inhibits autophagy through an inhibitory phosphorylation at Ser757 of ULK1 (Unc-51-like kinase 1), the kinase responsible for initiating autophagosome formation. The second is direct activation of ULK1 by AMPK through phosphorylation of ULK1 at Ser317 [103–105]. In addition to this function in global autophagy, ATM has recently been shown to mediate mitophagy and pexophagy, two specific types of autophagy targeting mitochondria and peroxisomes, respectively, following exposure to oxidative stress [106, 107]. Mitophagy and pexophagy induction by this mechanism could have implications in lowering cellular ROS (reactive oxygen species) levels following irradiation or other oxidative damaging therapies allowing for cancer cell resistance and survival to therapies of this nature [106, 107].

Another activator of autophagy following DNA damage is PARP1. PARP1, like AMPK, plays a bifunctional role in the activation of autophagy. The first is through its global activity following oxidative DNA damage in which it PARylates both DNA and proteins. This activity leads to the consumption of NAD^+ which eventually leads to a downstream depletion of ATP resulting in AMPK activation. As noted previously, AMPK activation leads to autophagy induction through negative regulation of mTOR through TSC1/2 and through positive regulation of the ULK1 [108]. In addition, PARP1 has recently been shown to be in complex with nuclear AMPK. Under starvation, a cellular state characterized by oxidative stress and DNA damage, nuclear AMPK is PARsylated and subsequently exported into the cytosol [109]. The importance of this event is critical as it allows for early activation of an autophagic response following DNA damage without the need for transcription to occur. Additionally, activation in this fashion does not affect the independent ATM activation of cytoplasmic AMPK [109]. In this way, the response is tailored to DDS through PARP1.

ATM and PARP1 represent just two components of the DDR in regulation of autophagy. Several other proteins involved in the DDR regulate autophagy either directly or indirectly. Examples include members of the sirtuin family of proteins (SIRT1–7) as well as FIP200 and SQSTM1/p62. While their influence on induction of autophagy is well documented, many of the roles carried out by these proteins regarding autophagy fall out of the realm of the DDR [110–113]. For this reason, interested readers are referred to the recent review by Czarny and Blasiak for more information [113].

6.3.2 Inflammation

Another cellular response elicited by the DDR is the induction of inflammatory cytokines through activation of NF-κB. Following various types of DNA damage, ATM and PARP1 play a synergistic role in the activation of NF-κB [114]. PARP1 functions to upregulate gene expression of NF-κB as well as in its direct activation. Upon DNA damage, PARP1 auto-PARylation serves as a signaling event for the recruitment of IKKγ as well as ATM and the E3-type small ubiquitin-like modifier (SUMO) ligase, PIASγ. This interaction leads to the SUMOylation of IKKγ by PIASγ at Lys277 and Lys309, resulting in IKKγ activation. Activated IKKγ stimulates NF-κB transcriptional activity and induction of proinflammatory cytokines promoting cellular survival and chemotherapeutic resistance [115]. Additionally, there is evidence that NF-κB is directly PARylated by PARP1; however, studies conflict on the exact consequence of this modification [115–117]. In addition to PARP1, ATM also plays a role in IKKγ activation through phosphorylation of IKKγ at Ser85. In conjunction with the SUMO modifications elicited by PIASγ, this promotes binding and activation of NF-κB [114]. NF-κB activity leads to the induction of BRCA2 and ATM transcription as well as the promotion of HR through enhanced DNA end resection following DSBs [118].

While the activation of NF-κB is protective, there are times were pathogenic induction of inflammation can occur following DNA damage. When exposed to chronic DNA damage, p53 is continuously activated by ATM, ATR, and DNA-PK. This can lead to the release of the proinflammatory protein HMGB1. HMGB1 has a variety of functions; however, in this context it is released as an extracellular damage-associated molecular pattern (DAMP). DAMPs activate macrophages and dendritic cells leading to the induction of TNFα, IL-1, and IL-6 [119]. This response causes a prolonged inflammatory state that can lead to both tissue injury as well as tumorigenesis [120, 121].

6.3.3 Cell Death

In cases of severe DNA damage, both cycling and postmitotic cells must have programs in place to ensure that unrepairable damaged cells do not persist to become cancerous. The most common way for cells to eliminate themselves when this occurs is to trigger apoptosis, a highly regulated and energy-dependent form of cell death. While there are many elicitors of apoptosis, this section will focus mainly on the apoptotic pathways promoted through p53 as it is the main effector protein in this process (Fig. 6.5a).

Following DNA damage and activation of apical kinases, ATM, ATR, and DNA-PK rapidly phosphorylate p53 at Ser15. While many other phosphorylation events occur upon p53, most serve to stabilize the protein and prevent it from rebinding to its negative regulator, MDM2. Ser15 phosphorylation serves to activate the

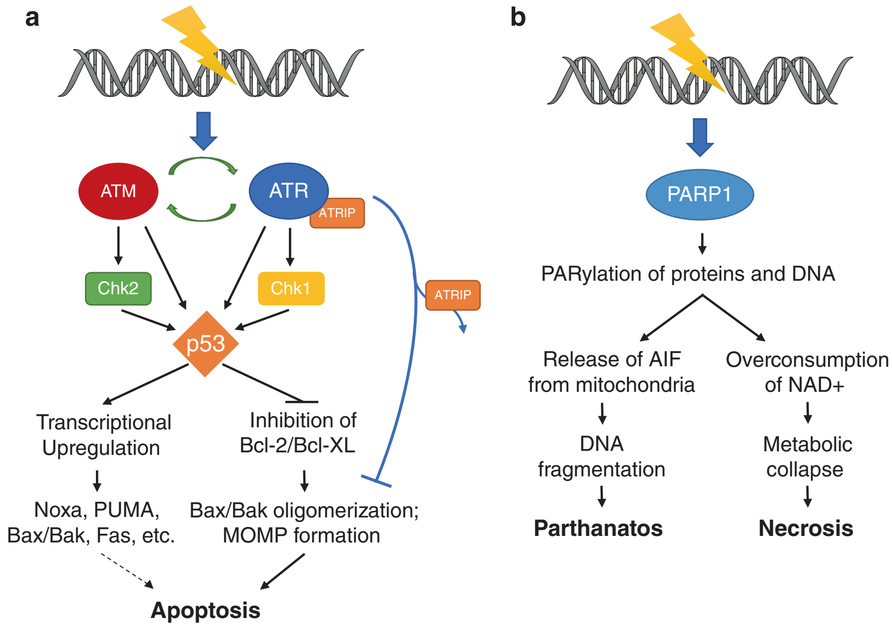

Fig. 6.5 DNA damage and cell death. Severe DNA damage can lead to the death of cells. This typically occurs through one of three mechanisms: (**a**) p53-mediated apoptosis or (**b**) PARP1-mediated parthanatos or necrosis. p53 can mediate apoptosis either directly or through induction of gene transcription where it upregulates proteins involved in both the intrinsic and extrinsic apoptosis pathways. In contrast to the regulated forms of death carried out by p53, PARP1 mediates two versions of cell death that have little order. The first is parthanatos which involves export of AIF from the mitochondria where it is then imported into the nucleus and causes non-specific DNA fragmentation. The other, necrosis, is highly unregulated and is based on the overconsumption of NAD^+ leading to decreased ATP production and metabolic collapse

transcriptional activity of p53, leading to upregulation of several pro-apoptotic genes: Bax/Bak, Puma, Noxa, Fas, etc. [122, 123]. Puma and Noxa, both members of the Bcl-2 family, serve to disrupt the binding of Bax and Bak to their respective negative regulators Bcl-2 and Bcl-XL. This allows for Bax and Bak to induce mitochondrial outer membrane permeabilization (MOMP) and the release of cytochrome c through self- and hetero-oligomerization in MOMP complex formation. These events lead to rapid apoptosome formation, culminating in apoptosis [124, 125]. Fas-ligand receptor, a pro-apoptotic receptor, also is upregulated upon p53 transcriptional activation. This and other upregulated pro-death receptors serve to facilitate p53-dependent extrinsic apoptosis following DNA damage [125]. This is in addition to the transcriptional activities of p53 listed previously in Sect. 2.1.

As well as transcriptional upregulation, p53 associates through its DNA-binding domain with mitochondria where it directly promotes MOMP. This occurs through p53 displacement of Bcl-2 and Bcl-XL from Bax and Bak, respectively, and is independent of p53's transcriptional activity. It is thought, in this way, to act as a pro-apoptotic BH3-like protein in its disruption of the anti-apoptotic functions of Bcl-2

and Bcl-XL [126, 127]. As p53 phosphorylation allows p53 to change conformation and bind DNA, it is possible that ATM, ATR, and DNA-PK DDS promote this direct apoptotic function. This, however, is not without regulation as both ATR and ATM also have both direct and indirect anti-apoptotic activities [128–130].

In addition to apoptosis, cell death following extreme DNA damage can occur via autosis, parthanatos, or necrosis. Autosis, or excessive autophagy, can lead to cell death through overstimulation of the autophagic mechanisms presented in Sect. 3.1 which leads to over catabolism of cellular components and metabolic collapse [131]. Parthanatos and necrosis are two cell death processes primarily dependent on PARP1 [1, 31]. In parthanatos, PARP1 induction of PARylation serves as a signal leading to the release of AIF (apoptosis-inducing factor) from the mitochondria and its import into the nucleus. Once in the nucleus, AIF cleaves DNA in a non-specific fashion leading to its degradation and resulting in cell death. Necrosis results from PARP1 overconsumption of NAD^+ leading to a deficiency of NAD^+, loss of glycolytic capability, and, ultimately, to metabolic collapse (Fig. 6.4b) [116].

6.4 Implication in Cancer: Chemotherapeutics and the DNA Damage Response

Given the importance of the DDR in processing DNA damage, it is imperative to consider the role of DDR in response to chemotherapy. Current chemotherapeutic mechanisms range from protein inhibition to DNA-damaging agents. These can target neoplasms dependent on genetic profile and origin. Due to the unique nature of each cancer, a variety of screening techniques have been developed to identify commonly occurring mutations allowing for more specific and targeted approaches to be applied [132, 133].

The DDR is invoked following various chemotherapeutic treatments. The simplest of which are those that lead to DNA damage (Table 6.2). These are the mainstay of most treatment regimens and have long been used to treat neoplastic malignancies. Agents in this category range from base-modifying agents (alkylators and cross-linkers) to direct and indirect strand break inducers (antimetabolites, topoisomerase inhibitors, mitotic inhibitors, and radiomimetics). These trigger the DDR processes detailed previously within this chapter [133, 134]. These DNA-damaging agents, pathways activated, and repair types initiated are summarized in Table 6.2 for ease of reference.

While significantly effective treatments, DNA damage therapies are typically highly toxic. In most instances, this toxicity is not limited to just cancer cells, but also affects non-cancerous cells as well. Moreover, it is common for cancers to develop a resistance to direct DNA-damaging agents alone over the course of treatment due to acquired mutations [132, 133]. Because of this, there is a constant need for development of alternative strategies for treatment. One way forward is through profiling of mutations leading to deficiencies in various DDR pathways.

Many DDR proteins commonly contain mutations contributing to carcinogenesis. While this contribution can be through loss- or gain-of-function, the outcome is

Table 6.2 DNA-damaging chemotherapeutics and DDR

Chemotherapeutic class (representative drug)	DNA damage induced	DNA damage response pathway activated	DNA repair type
Alkylators – Alkyl sulfonates (busulfan) – Ethylenimine (altretamine, thiotepa) – Nitrogen mustards (cyclophosphamide, ifosfamide) – Nitrosoureas (lomustine, streptozocin) – Triazines (dacarbazine, temozolomide)	DNA alkylation	ATM/ATR	BER, MMR, NER
Antimetabolites/nucleoside analogues – 5-Fluorouracil – Cytarabine – Gemcitabine	Mismatch, replication stress (SSB, DSB)	ATR/ATM	BER, HR, MMR
Cross-linkers – Antitumor antibiotics (mitomycin-C) – Platinums (cisplatin, carboplatin)	Inter- and intrastrand cross-links	ATR/ATM	NER, ICLR
Topoisomerase inhibitors – Topoisomerase I (topotecan, irinotecan [CPT-11]) – Topoisomerase II (etoposide [VP-16], teniposide) – Topoisomerase II (anthracycline antibiotic, doxorubicin) – Topoisomerase II (anthraquinone antibiotic, mitoxantrone)	SSB, DSB, protein-DNA topo-adducts	ATR/ATM	NER, HR, NHEJ
Mitotic inhibitors – Taxanes (pacitaxel, docetaxel) – Epothilones (ixabepilone) – Vinca alkaloids (vinblastine, vincristine)	Mitotic collapse leading to DSB formation	ATM/ATR/DNA-PK	HR, NHEJ
Radiomimetics – Antitumor antibiotics (bleomycin, C-1027)	SSB, DSB, base oxidation	ATM/ATR/DNA-PK	BER, HR, NHEJ

always an alteration of underlying pathways resulting in genomic instability. Although these mutations, and subsequent genomic instability, contributed to carcinogenesis, they also can be exploited therapeutically. Synthetic lethality approaches aim to increase toxicity of chemotherapeutics to neoplastic cells while simultaneously reducing toxicity in non-cancerous cells by exploiting these genetic deficiencies. In many cases, some individual therapeutics without adjuvant DNA-damaging treatments can be sufficient to induce cancer cell death due to underlying genetic deficiencies [132, 134].

A good example of the synthetic lethality approach is the use of PARP1 inhibitors in BRAC1- or BRAC2-deficient cancers. Inhibition of PARP1 leads to a deficiency in BER and subsequent accumulation of SSBs either with the PARP1 inhibitor alone or in combination with additional DNA-damaging agents. This leads to persistence of SSBs, resulting in DSBs and replication fork collapse during S phase. As mentioned previously, BRAC1 and BRAC2 are necessary for HR, and, as such, without functioning BRAC1/2, HR will not occur. Thus, tumors possessing mutations in these proteins are deficient in HR and must rely on other forms of DNA repair to maintain genomic integrity when DSBs and replication collapse occur. PARP1 inhibition also leads to reduced NHEJ and an absence of alt-NHEJ, resulting in a mass accumulation of DNA damage as cells lack the ability to repair the damage. Sustained damage in this fashion leads not only to failed DNA repair, but in many instances to cancer cell death [132–134]. To explore this concept, Li et al. recently reported a way to induce “BRCAness” together with PARP inhibition to produce synthetic lethality to non-BRCA-deficient drug-resistant prostate cancers [135].

Additional synthetic lethality approaches involving the DDR are currently under investigation. One such example is ATR inhibition in combination with either ionizing radiation or cross-linking agents. Inhibition of ATR leads to a lack of cell cycle checkpoint activation as well as direct failure of HR/ICLR following DNA damage. Furthermore,

Table 6.3 Role of current and potential chemotherapeutics targeting DDR

Role of current and potential chemotherapeutics targeting DDR		
Chemotherapeutic class (representative drug)	Cellular pathway affected	Effect on DNA damage response and signaling
Checkpoint kinase inhibitors		
– Chk1 (LY2603618, MK-8776) – Chk2 (PV1019, VRX046617)[a]	ATR ATM	Cell cycle progression (mutation accumulation, mitotic catastrophe, and potentiation of DNA damage), reduced activation of Rad51 leading to HR deficiency Cell cycle progression (mutation accumulation, replication stress, and potentiation of DNA damage), reduced activation of BRAC1/2 leading to inefficient HR
Cyclin-dependent kinase inhibitors		
– CDK1 – CDK2 – CDK4/6 – pan-CDK	CCA at G2/M CCA at G1/S CCA in G1 Complete CCA	Potentiates cell cycle arrest induced by concurrent chemotherapeutic treatments, allows for the accumulation of DNA damage and pro-apoptotic signaling
DNA ligase IV inhibitor[a]		
– L189, SCR7	NHEJ	Inhibition of DNA ligase IV preventing ligation following NHEJ, prolongation of DSBs and increased ATM signaling
HDAC inhibitors[b]		

Table 6.3 (continued)

Role of current and potential chemotherapeutics targeting DDR		
Chemotherapeutic class (representative drug)	Cellular pathway affected	Effect on DNA damage response and signaling
– Class I (HDAC 1,2,3,8) – Class IIB (HDAC 6) – Class III (SIRT1, 6, 7)	NER, NHEJ, p53 MMR BER, HR, NER, Alt-NHEJ, p53	HR and NHEJ, accumulation of H3K56Ac, H4K16A, H4K91Ac preventing protein recruitment; p53, simulation of p53 transcription MMR, increases MSH2 stability leading to better detection of mismatched bases BER, controls substrate specificity of TDG and lessens APE1 activity (Sirt1), lessened activation of WRN(Sirt1), lessened activation of PARP1 (Sirt6); HR, activation of CtIP; HR/Alt-NHEJ, lessened activation of PARP1 (Sirt6); NER, lessened XPA binding to other NER factors (Sirt1); p53, potentiated p53-induced apoptosis (Sirt1)
Ku70/Ku80 inhibitors[a]		
– Vitas-M STL127705, ZINC 09009828	NHEJ	Prevention of Ku70/80 binding to DNA leading to loss of NHEJ function
MRN inhibitor[a]		
– Mirin – PFM01, PFM03, PFM39	HR, NHEJ (minor) HR	Failure of MRN activation of ATM (loss of ATM dependent signaling), inhibition of MRE11 nuclease activity Inhibition of MRE11 nuclease activity, promotion of NHEJ
RAD51 inhibitor[a]		
– B02, DIDS, RI-1, RI-2 – IBR2, IBR120	HR HR	Inhibition of RAD51 ssDNA-binding activity Inhibition of RAD51 binding to BRAC2, decrease in BRAC2 recruitment to sites of DNA damage
RPA[a]		
– TDRL551	HR, ICLR, NER	Disruption of ssDNA-binding capacity and replication, replication fork collapse, lessened recruitment of ATR-ATRIP
PIKK family inhibitors		
– ATM (KU-55933, KU-60019)[a] – ATR (AZD3738, VE-821, VE-822/ VX-970) – DNA-PK (NU7026, KU-0060648)	HR, ATM HR, ICLR, ATR NHEJ	Increased accumulation of DNA damage due to loss of cell cycle control, inhibition of HR, reduced autophagic signaling following DNA damage Increased accumulation of DNA damage due to loss of cell cycle control, loss of HR and ICLR leading to increased strand breakage and NHEJ Inhibition of NHEJ forcing the use of HR
PARP1 inhibitors		
– Oliparib, Rucaparib, Veliparib	Alt-NHEJ, BER, HR (minor), NHEJ	Alt-NHEJ and BER, failure to recruit XRCC1; HR, lessened recruitment of MRE11 and RAD51 to facilitate stalled replication fork restart; NHEJ, lessened DNA-PK activation and failure to recruit DNA ligase IV

[a]No inhibitors of this type have reached the clinical trial stage
[b]HDAC class IIA shows little effect on the DDR

inhibition of ATR forces the use of the NHEJ pathway which can lead to further accumulation of deleterious mutations as it is not a high-fidelity form of repair [136].

Many other inhibitors targeting the DDR are under development or already have reached clinical trial (Table 6.3) [132–134, 136–140]. As can be noted from Table 6.3, almost every aspect of the DDR is currently under investigation. With advanced technologies increasing tumor profiling capability, there likely will be a rise in synthetic lethality approaches using DDR proteins as targets. This will hopefully lead to increased chemotherapeutic efficiency as well as reduce off-target toxicity; both of which are essential for good patient outcome.

References

1. Ciccia A, Elledge SJ. The DNA damage response: making it safe to play with knives. Mol Cell. 2010;40(2):179–204.
2. Sirbu BM, Cortez D. DNA damage response: three levels of DNA repair regulation. Cold Spring Harb Perspect Biol. 2013;5(8):a012724.
3. Marechal A, Zou L. DNA damage sensing by the ATM and ATR kinases. Cold Spring Harb Perspect Biol. 2013;5(9).
4. Sancar A, et al. Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. Annu Rev Biochem. 2004;73:39–85.
5. Fell VL, et al. Ku70 Serine 155 mediates Aurora B inhibition and activation of the DNA damage response. Sci Rep. 2016;6(37194):37194.
6. Zhou Y, et al. Regulation of the DNA damage response by DNA-PKcs inhibitory phosphorylation of ATM. Mol Cell. 2017;65(1):91–104.
7. Bakkenist CJ, Kastan MB. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature. 2003;421(6922):499–506.
8. Kozlov SV, et al. Autophosphorylation and ATM activation: additional sites add to the complexity. J Biol Chem. 2011;286(11):9107–19.
9. Falck J, et al. The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. Nature. 2001;410(6830):842–7.
10. Mailand N, et al. Rapid destruction of human Cdc25A in response to DNA damage. Science. 2000;288(5470):1425–9.
11. Abbas T, Dutta A. p21 in cancer: intricate networks and multiple activities. Nat Rev Cancer. 2009;9(6):400–14.
12. Maya R, et al. ATM-dependent phosphorylation of Mdm2 on serine 395: role in p53 activation by DNA damage. Genes Dev. 2001;15(9):1067–77.
13. Wu X, Shell SM, Zou Y. Interaction and colocalization of Rad9/Rad1/Hus1 checkpoint complex with replication protein A in human cells. Oncogene. 2005;24(29):4728–35.
14. Cortez D, et al. ATR and ATRIP: partners in checkpoint signaling. Science. 2001;294(5547):1713–6.
15. Zou L, Elledge SJ. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science. 2003;300(5625):1542–8.
16. Liu S, et al. ATR autophosphorylation as a molecular switch for checkpoint activation. Mol Cell. 2011;43(2):192–202.
17. Kumagai A, et al. TopBP1 activates the ATR-ATRIP complex. Cell. 2006;124(5):943–55.
18. Mordes DA, et al. TopBP1 activates ATR through ATRIP and a PIKK regulatory domain. Genes Dev. 2008;22(11):1478–89.
19. Lee J, Kumagai A, Dunphy WG. The Rad9-Hus1-Rad1 checkpoint clamp regulates interaction of TopBP1 with ATR. J Biol Chem. 2007;282(38):28036–44.

20. Zhou XY, et al. An ATM-independent S-phase checkpoint response involves CHK1 pathway. Cancer Res. 2002;62(6):1598–603.
21. Bartek J, Lukas J. Chk1 and Chk2 kinases in checkpoint control and cancer. Cancer Cell. 2003;3(5):421–9.
22. Yang Q, et al. Identification of a functional domain in a GADD45-mediated G2/M checkpoint. J Biol Chem. 2000;275:36892–8.
23. Peng C. Mitotic and G2 checkpoint control: regulation of 14-3-3: protein binding by phosphorylation of Cdc25C on Serine-216. Science. 1997;277(5331):1501–5.
24. Harvey SL, et al. Cdk1-dependent regulation of the mitotic inhibitor Wee1. Cell. 2005;122(3):407–20.
25. Zitouni S, et al. Polo-like kinases: structural variations lead to multiple functions. Nat Rev Mol Cell Biol. 2014;15(7):433–52.
26. Mackay DR, Ullman KS. ATR and a Chk1-Aurora B pathway coordinate postmitotic genome surveillance with cytokinetic abscission. Mol Biol Cell. 2015;26(12):2217–26.
27. Myers JS, Cortez D. Rapid activation of ATR by ionizing radiation requires ATM and Mre11. J Biol Chem. 2006;281(14):9346–50.
28. Stiff T, et al. ATR-dependent phosphorylation and activation of ATM in response to UV treatment or replication fork stalling. EMBO J. 2006;25(24):5775–82.
29. Matsuoka S, et al. ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. Science. 2007;316(5828):1160–6.
30. Serrano MA, et al. DNA-PK, ATM and ATR collaboratively regulate p53-RPA interaction to facilitate homologous recombination DNA repair. Oncogene. 2013;32(19):2452–62.
31. Wei H, Yu X. Functions of PARylation in DNA damage repair pathways. Genomics Proteomics Bioinformatics. 2016;14(3):131–9.
32. Roos WP, Krumm A. The multifaceted influence of histone deacetylases on DNA damage signaling and DNA repair. Nucleic Acids Res. 2016;44(21):10017–30.
33. Maiti A, Drohat AC. Thymine DNA glycosylase can rapidly excise 5-formylcytosine and 5-carboxylcytosine: potential implications for active demethylation of CpG sites. J Biol Chem. 2011;286:35334–8.
34. Madabushi A, et al. Histone deacetylase SIRT1 modulates and deacetylates DNA base excision repair enzyme thymine DNA glycosylase. Biochem J. 2013;456(1):89–98.
35. Yamamori T, et al. SIRT1 deacetylates APE1 and regulates cellular base excision repair. Nucleic Acids Res. 2010;38(3):832–45.
36. Harrigan JA, et al. The Werner syndrome protein stimulates DNA polymerase beta strand displacement synthesis via its helicase activity. J Biol Chem. 2003;278(25):22686–95.
37. Li K, et al. Regulation of WRN protein cellular localization and enzymatic activities by SIRT1-mediated deacetylation. J Biol Chem. 2008;283(12):7590–8.
38. Scharer OD. Nucleotide excision repair in eukaryotes. Cold Spring Harb Perspect Biol. 2013;5(10):a012609.
39. Mu D, Sancar A. Model for XPC-independent transcription-coupled repair of pyrimidine dimers in humans. J Biol Chem. 1997;272(12):7570–3.
40. Sugasawa K, et al. Xeroderma pigmentosum group C protein complex is the initiator of global genome nucleotide excision repair. Mol Cell. 1998;2(2):223–32.
41. Fitch ME, et al. In vivo recruitment of XPC to UV-induced cyclobutane pyrimidine dimers by the DDB2 gene product. J Biol Chem. 2003;278(47):46906–10.
42. Marteijn JA, Hoeijmakers JH, Vermeulen W. Check, Check ...Triple Check: multi-step DNA lesion identification by nucleotide excision repair. Mol Cell. 2015;59(6):885–6.
43. Li C, et al. Tripartite DNA lesion recognition and verification by XPC, TFIIH, and XPA in nucleotide excision repair. Mol Cell. 2015;59(6):1025–34.
44. O'Donovan A, et al. XPG endonuclease makes the 3′ incision in human DNA nucleotide excision repair. Nature. 1994;371(6496):432–5.
45. Gaillard PH, Wood RD. Activity of individual ERCC1 and XPF subunits in DNA nucleotide excision repair. Nucleic Acids Res. 2001;29(4):872–9.

46. Gaillard H, Aguilera A. Transcription coupled repair at the interface between transcription elongation and mRNP biogenesis. Biochim Biophys Acta. 2013;1829:141–50.
47. Fan W, Luo J. Sirt1 regulates UV-induced DNA repair through deacetylating XPA. Mol Cell. 2010;39:247–58.
48. Lee TH, et al. Coordinated regulation of XPA stability by ATR and HERC2 during nucleotide excision repair. Oncogene. 2014;33(1):19–25.
49. Duckett DR, et al. Human MutSAlpha recognizes damaged DNA base pairs continaing 06-methylguanine, O4-methylthymine, or the cisplatin-d(GpG) adduct. Proc Natl Acad Sci U S A. 1996;93:6443–7.
50. Christmann M, et al. Mechanisms of human DNA repair: an update. Toxicology. 2003;193(1–2):3–34.
51. Wang M, et al. PARP-1 and Ku compete for repair of DNA double strand breaks by distinct NHEJ pathways. Nucleic Acids Res. 2006;34(21):6170–82.
52. Goodarzi AA, et al. DNA-PK autophosphorylation facilitates Artemis endonuclease activity. EMBO J. 2006;25(16):3880–9.
53. Goodwin JF, Knudsen KE. Beyond DNA repair: DNA-PK function in cancer. Cancer Discov. 2014;4(10):1126–39.
54. Neal JA, Meek K. Choosing the right path: does DNA-PK help make the decision. Mutat Res. 2011;711:73–86.
55. Lopez-Contreras AJ, Fernandez-Capetillo O. Signalling DNA damage. INTECH. 2012;8:233–62.
56. Li M, et al. The FHA and BRCT domains recognize ADP-ribosylation during DNA damage response. Genes Dev. 2013;27(16):1752–68.
57. Deriano L, Roth DB. Modernizing the nonhomologous end-joining repertoire: alternative and classical NHEJ share the stage. Annu Rev Genet. 2013;47:433–55.
58. Jackson SP, Durocher D. Regulation of DNA damage responses by ubiquitin and SUMO. Mol Cell. 2013;49(5):795–807.
59. Sartori AA, et al. Human CtIP promotes DNA end resection. Nature. 2007;450(7169):509–14.
60. Nimonkar AV, et al. Human exonuclease 1 and BLM helicase interact to resect DNA and initiate DNA repair. Proc Natl Acad Sci U S A. 2008;105(44):16906–11.
61. Paull TT, Gellert M. The 3′ to 5′ exonuclease activity of Mre 11 facilitates repair of DNA double-strand breaks. Mol Cell. 1998;1(7):969–79.
62. DiTullio RA, et al. 53BP1 functions in an ATM-dependent checkpoint pathway that is constitutively activated in human cancer. Nat Cell Biol. 2002;4(12):998–1002.
63. Savage KI, Harkin DP. BRCA1, a 'complex' protein involved in the maintenance of genomic stability. FEBS J. 2015;282(4):630–46.
64. Gudmundsdottir K, Ashworth A. The roles of BRCA1 and BRCA2 and associated proteins in the maintenance of genomic stability. Oncogene. 2006;25(43):5864–74.
65. Fradet-Turcotte A, et al. BRCA2 functions: from DNA repair to replication fork stabilization. Endocr Relat Cancer. 2016;23(10):T1–T17.
66. Constantinou A, et al. Holliday junction resolution in human cells: two junction endonucleases with distinct substrate specificities. EMBO J. 2002;21(20):5577–85.
67. Wu L, Hickson ID. The Bloom's syndrome helicase suppresses crossing over during homologous recombination. Nature. 2003;426(6968):870–4.
68. Svendsen JM, Harper JW. GEN1/Yen1 and the SLX4 complex: solutions to the problem of Holliday junction resolution. Genes Dev. 2010;24(6):521–36.
69. Kaidi A, et al. Human SIRT6 promotes DNA end resection through CtIP deacetylation. Science. 2010;329(5997):1348–53.
70. Clauson C, Scharer OD, Niedernhofer L. Advances in understanding the complex mechanisms of DNA interstrand cross-link repair. Cold Spring Harb Perspect Biol. 2013;5(10):a012732.
71. Gari K, et al. The Fanconi anemia protein FANCM can promote branch migration of Holliday junctions and replication forks. Mol Cell. 2008;29(1):141–8.

72. Huang M, et al. The FANCM/FAAP24 complex is required for the DNA interstrand crosslink-induced checkpoint response. Mol Cell. 2010;39(2):259–68.
73. Meetei AR, Yan Z, Wang W. FANCL replaces BRCA1 as the likely ubiquitin ligase responsible for FANCD2 monoubiquitination. Cell Cycle. 2004;3(2):179–81.
74. Krishnan V, Tay LS, Ito Y. The Fanconi anemia pathway of DNA repair and human cancer. In: Chen CC, editor. Advances in DNA repair. Rijeka, Croatia: InTech; 2015. p. 255–89.
75. Singh TR, et al. ATR-dependent phosphorylation of FANCM at serine 1045 is essential for FANCM functions. Cancer Res. 2013;73(14):4300–10.
76. Ho GP, et al. Phosphorylation of FANCD2 on two novel sites is required for mitomycin C resistance. Mol Cell Biol. 2006;26(18):7005–15.
77. Ishiai M, et al. FANCI phosphorylation functions as a molecular switch to turn on the Fanconi anemia pathway. Nat Struct Mol Biol. 2008;15(11):1138–46.
78. Wang X, et al. Chk1-mediated phosphorylation of FANCE is required for the Fanconi anemia/BRCA pathway. Mol Cell Biol. 2007;27(8):3098–108.
79. Collins NB, et al. ATR-dependent phosphorylation of FANCA on serine 1449 after DNA damage is important for FA pathway function. Blood. 2009;113(10):2181–90.
80. Mjelle R, et al. Cell cycle regulation of human DNA repair and chromatin remodeling genes. DNA Repair. 2015;30:53–67.
81. Warmerdam DO, Kanaar R. Dealing with DNA damage: relationships between checkpoint and repair pathways. Mutat Res. 2010;704(1–3):2–11.
82. Hustedt N, Durocher D. The control of DNA repair by the cell cycle. Nat Cell Biol. 2016;19(1):1–9.
83. Wu X, et al. Phosphorylation of nucleotide excision repair factor xeroderma pigmentosum group A by ataxia telangiectasia mutated and Rad3-related-dependent checkpoint pathway promotes cell survival in response to UV irradiation. Cancer Res. 2006;66(6):2997–3005.
84. Shell SM, et al. Checkpoint kinase ATR promotes nucleotide excision repair of UV-induced DNA damage via physical interaction with xeroderma pigmentosum group A. J Biol Chem. 2009;284(36):24213–22.
85. Li Z, et al. UV-induced nuclear import of XPA is mediated by importin-alpha4 in an ATR-dependent manner. PLoS One. 2013;8(7):e68297.
86. Wu X, et al. ATR-dependent checkpoint modulates XPA nuclear import in response to UV irradiation. Oncogene. 2007;26(5):757–64.
87. Jarrett SG, et al. PKA-mediated phosphorylation of ATR promotes recruitment of XPA to UV-induced DNA damage. Mol Cell. 2014;54(6):999–1011.
88. Li Z, et al. XPA-mediated regulation of global nucleotide excision repair by ATR is p53-dependent and occurs primarily in S-phase. PLoS One. 2011;6(12):e28326.
89. Barckhausen C, et al. Malignant melanoma cells acquire resistance to DNA interstrand cross-linking chemotherapeutics by p53-triggered upregulation of DDB2/XPC-mediated DNA repair. Oncogene. 2014;33(15):1964–74.
90. Christmann M, et al. Adaptive upregulation of DNA repair genes following benzo(a)pyrene diol epoxide protects against cell death at the expense of mutations. Nucleic Acids Res. 2016;44(22):10727–43.
91. Wang XW, et al. p53 modulation of TFIIH-associated nucleotide excision repair activity. Nat Genet. 1995;10(2):188–95.
92. Wang QE, et al. Tumor suppressor p53 dependent recruitment of nucleotide excision repair factors XPC and TFIIH to DNA damage. DNA Repair. 2003;2(5):483–99.
93. Zhou J, et al. A role for p53 in base excision repair. EMBO J. 2001;20(4):914–23.
94. Zurer I, et al. The role of p53 in base excision repair following genotoxic stress. Carcinogenesis. 2004;25(1):11–9.
95. Romanova LY, et al. The interaction of p53 with replication protein A mediates suppression of homologous recombination. Oncogene. 2004;23(56):9025–33.

96. Yu X, et al. The C-terminal (BRCT) domains of BRCA1 interact in vivo with CtIP, a protein implicated in the CtBP pathway of transcriptional repression. J Biol Chem. 1998;273(39):25388–92.
97. Huertas P, Jackson SP. Human CtIP mediates cell cycle control of DNA end resection and double strand break repair. J Biol Chem. 2009;284(14):9558–65.
98. Steger M, et al. Prolyl isomerase PIN1 regulates DNA double-strand break repair by counteracting DNA end resection. Mol Cell. 2013;50(3):333–43.
99. Klionsky DJ, Emr SD. Autophagy as a regulated pathway of cellular degradation. Science. 2000;290:1717–21.
100. Abedin MJ, et al. Autophagy delays apoptotic death in breast cancer cells following DNA damage. Cell Death Diff. 2007;14:500–10.
101. Elliott A, Reiners JJ Jr. Suppression of autophagy enhances the cytotoxicity of the DNA-damaging aromatic amine p-anilinoaniline. Toxicol Appl Pharmacol. 2008;232(2):169–79.
102. Apel A, et al. Blocked autophagy sensitizes resistant carcinoma cells to radiation therapy. Cancer Res. 2008;68(5):1485–94.
103. Alexander A, Kim J, Walker CL. ATM engages the TSC2/mTORC1 signaling node to regulate autophagy. Autophagy. 2010;6(5):672–3.
104. Zhao M, Klionsky DJ. AMPK-dependent phosphorylation of ULK1 induces autophagy. Cell Metab. 2011;13:119–20.
105. Tripathia DN, et al. Reactive nitrogen species regulate autophagy through ATM-AMPK-TSC2–mediated suppression of mTORC1. PNAS. 2013;110:E2950–E7.
106. Zhang J, et al. ATM functions at the peroxisome to induce pexophagy in response to ROS. Nat Cell Biol. 2015;17(10):1259–69.
107. Valentin-Vega YA, Kastan MB. A new role for ATM regulating mitochondrial function and mitophagy. Autophagy. 2012;5:840–1.
108. Hoyer-Hansen M, Jaattela M. AMP-activated protein kinases: a universal regulator of autophagy? Autophagy. 2007;3:381–3.
109. Rodrigues-Vargas JM, et al. Autophagy requires poly(adp-ribosyl)ation-dependent AMPK nuclelar export. Cell Death Diff. 2016;23:2007–18.
110. Yoon JH, et al. Role of autophagy in chemoresistance: regulation of the ATM-mediated DNA-damage signaling pathway through activation of DNA-PKcs and PARP-1. Biochem Pharmacol. 2012;83:747–57.
111. Rajendran R, et al. Sirtuins: molecular traffic lights in the crossroad of oxidative stress, chromatin remodeling, and transcription. J Biomed Biotechnol. 2011;2011:1–17.
112. Pankiv S, et al. Nucleocytoplasmic shuttling of p62/SQSTM1 and its role in recruitment of nuclear polyubiquitinated proteins to promyelocytic leukemia bodies. J Biol Chem. 2010;283:6783–9.
113. Czarny P, et al. Autophagy and the DNA damage response. Int J Mol Sci. 2015;16:2641–62.
114. Miyamoto S. Nuclear initiated NF-κB signaling: NEMO and ATM take center stage. Cell Res. 2011;21:116–30.
115. Veuger SJ, Hunter JE, Durkacz BW. Ionizing radiation-induced NF-kappaB activation requires PARP-1 function to confer radioresistance. Oncogene. 2009;28:832–42.
116. Weaver AN, Yang ES. Beyond DNA repair: additional functions of PARP-1 in cancer. Front Oncol. 2013;3:290.
117. Chang WJ, Alvarez-Gonzalez R. The sequence-specific DNA binding of NFkappa B is reversibly regulated by the automodification reaction of poly (ADP ribose) polymerase 1. J Biol Chem. 2001;276:47664–70.
118. Volcic M, et al. NF-kappaB regulates DNA double-strand break repair in conjunction with BRCA1-CtIP complexes. Nucleic Acids Res. 2012;40(1):181–95.
119. Andersson U, et al. High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes. J Exp Med. 2000;192(4):565–70.
120. Kodama T, et al. Increases in p53 expression induce CTGF synthesis by mouse and human hepatocytes and result in liver fibrosis in mice. J Clin Invest. 2011;121(8):3343–56.

121. Yan HX, et al. p53 promotes inflammation-associated hepatocarcinogenesis by inducing HMGB1 release. J Hepatol. 2013;59(4):762–8.
122. Riley T, et al. Transcriptional control of human p53-regulated genes. Nat Rev Mol Cell Biol. 2008;9(5):402–12.
123. Zilfou JT, Lowe SW. Tumor suppressive functions of p53. Cold Spring Harb Perspect Biol. 2009;1(5):a001883.
124. Vaseva AV, Moll UM. The mitochondrial p53 pathway. Biochim Biophys Acta. 2009;1787(5):414–20.
125. Brady CA, Attardi LD. p53 at a glance. J Cell Sci. 2010;123(15):2527–32.
126. Leu JI, et al. Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex. Nat Cell Biol. 2004;6(5):443–50.
127. Chipuk JE, et al. PUMA couples the nuclear and cytoplasmic proapoptotic function of p53. Science. 2005;309(5741):1732–5.
128. Korwek Z, et al. Inhibition of ATM blocks the etoposide-induced DNA damage response and apoptosis of resting human T cells. DNA Repair (Amst). 2012;11(11):864–73.
129. Imanishi S, et al. Constitutive activation of the ATM/BRCA1 pathway prevents DNA damage-induced apoptosis in 5-azacytidine-resistant cell lines. Biochem Pharmacol. 2014;89(3):361–9.
130. Hilton BA, et al. ATR plays a direct antiapoptotic role at mitochondria, which is regulated by prolyl isomerase Pin1. Mol Cell. 2015;60(1):35–46.
131. Surova O, Zhivotovsky B. Various modes of cell death induced by DNA damage. Oncogene. 2013;32:789–3797.
132. McLornan DP, List A, Mufti GJ. Applying synthetic lethality for the selective targeting of cancer. N Engl J Med. 2014;371(18):1725–35.
133. O'Connor MJ. Targeting the DNA damage response in cancer. Mol Cell. 2015;60(4):547–60.
134. Gavande NS, et al. DNA repair targeted therapy: the past or future of cancer treatment? Pharmacol Ther. 2016;160:65–83.
135. Li L, et al. Androgen receptor inhibitor-induced "BRCAness" and PARP inhibition are synthetically lethal for castration-resistant prostate cancer. Sci Signal. 2017;10(480).
136. Weber AM, Ryan AJ. ATM and ATR as therapeutic targets in cancer. Pharmacol Ther. 2015;149:124–38.
137. Mishra AK, et al. Chemical inhibitor targeting the replication protein A-DNA interaction increases the efficacy of Pt-based chemotherapy in lung and ovarian cancer. Biochem Pharmacol. 2015;93(1):25–33.
138. Velic D, et al. DNA damage signalling and repair inhibitors: the long-sought-after Achilles' heel of cancer. Biomol Ther. 2015;5(4):3204–59.
139. Weterings E, et al. A novel small molecule inhibitor of the DNA repair protein Ku70/80. DNA Repair (Amst). 2016;43:98–106.
140. Woods D, Turchi JJ. Chemotherapy induced DNA damage response: convergence of drugs and pathways. Cancer Biol Ther. 2013;14(5):379–89.

Chapter 7
Met Activation and Carcinogenesis

Nariyoshi Shinomiya, Qian Xie, and George F. Vande Woude

Abstract MET is a tyrosine kinase receptor that transduces intracellular signaling to activate the MAPK, PI3K-Akt, and cadherin pathways (among others). In cancer cells, MET is activated upon stimulation by its only ligand, hepatocyte growth factor/scatter factor (HGF/SF), or becomes active due to mutations or amplifications that produce constitutive activation of the MET kinase. The biological consequences of HGF/SF-MET signaling include cell proliferation, cell cycle progression, increased cell motility and invasive activity, and degradation of extracellular matrices, which can lead to oncogenesis. Aberrant MET signaling contributes to the carcinogenesis of hereditary cancers and also plays a major role in the spread of cancer cells; such signaling indicates a poor prognosis for cancer patients. Genetically engineered mouse models are important tools for studying the spontaneous development of tumors mediated by HGF/SF-MET signaling. Such tumors include carcinomas, sarcomas, and lymphomas, demonstrating the breadth of MET signaling as driving force of cancer. In this chapter, we will discuss the role of HGF/SF-MET signaling in carcinogenesis and the animal models used in developing therapeutic strategies that target the HGF/SF-MET signaling pathways.

Keywords Met/MET • HGF/SF • Carcinogenesis • Epithelial–mesenchymal transition (E–MT) • Dysregulation • Constitutive activation • Genetically engineered mouse models

N. Shinomiya (✉)
Department of Integrative Physiology and Bio-Nano Medicine, National Defense Medical College, Tokorozawa, Saitama, Japan
e-mail: shinomi@ndmc.ac.jp

Q. Xie
Department of Biomedical Sciences, Center of Excellence for Inflammation, Infectious Disease and Immunity, Quillen College of Medicine, East Tennessee State University, Johnson, TN, USA

G.F. Vande Woude
Distinguished Scientific Fellow, Emeritus, Van Andel Research Institute, Grand Rapids, MI, USA

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N. Shinomiya et al. (eds.), *Regulation of Signal Transduction in Human Cell Research*, Current Human Cell Research and Applications,
https://doi.org/10.1007/978-981-10-7296-3_7

Abbreviations

MET human MET
Met mouse Met

7.1 Introduction

Historically, MET was discovered as the *Trp-MET* oncogene and the *MET* proto-oncogene, and molecular characterization was performed in 1986 [1]. One year later, a fibroblast-derived protein which caused the scattering of epithelial cells was found and named "scatter factor" (SF) [2]; it was later identified as a ligand of MET. In 1989, human hepatocyte growth factor (HGF) was first cloned [3], and its nucleotide sequence revealed that it was identical to SF and that both α- and β-chains were contained in a single open reading frame. In the middle of the 1990s, the relationship between HGF/SF and Met was clarified by using knockout (KO) mouse models. Using Met KO mice, Met was proved to have an essential role in the migration of myogenic precursor cells into the limb bud and diaphragm [4]. As a result, skeletal muscles of the limb and diaphragm did not form in the KO mice, and they died as embryos. Similarly, embryonic death with placental defects was observed in mice lacking HGF/SF [5], and HGF/SF was found to be essential for the development of important epithelial organs such as the liver [6]. Because the phenotypes are quite similar between Met KO mice and HGF/SF KO mice [7], HGF/SF is considered to be the only ligand for Met, and Met the only receptor for HGF/SF.

HGF/SF-MET signaling also regulates a wide range of cancer cell functions such as proliferation, cell cycle progression, and control of the expression of adhesion molecules that induce extracellular matrix activation, migration, invasion, and neovasculogenesis (Chap. 8).

To clarify the function of MET and its downstream signals, extensive experiments were performed using normal as well as tumor cells. Upon stimulation by HGF/SF, MET is phosphorylated, which initiates downstream signal. The phosphorylation of tyrosine 1234/1235 in the MET kinase domain is crucial to kinase activation. The phosphorylation of tyrosine 1349 and tyrosine 1356 in the C-terminal region provides a direct binding capability to GRB2 (growth factor receptor-bound protein 2) and GAB1 (GRB2-associated binder 1), which transduce subsequent downstream signals [8, 9]. Both the Gab1–Shp2–ERK/MAPK and Grb2–Ras–Raf–ERK/MAPK pathways stimulate cell cycle regulators to induce cell proliferation and cell cycle progression. The activation of extracellular matrix proteinases alters cytoskeletal functions that control migration, invasion, and proliferation. Ras–Rac1/Cdc42–PAK activation disrupts E-cadherin adhesion [10], which affects cell polarity and actin cytoskeleton remodeling and enhances cell motility [11]. Gab1–Crk–C3G–Rap1 activation regulates paxillin, focal adhesion kinase, and integrins, and it loosens cell junctions, which leads to cell migration and invasion [12]. Activation of

GAB1–phosphatidylinositol 3-kinase (PI3K)–Akt/PKB pathway controls cell survival through the inhibition of apoptosis-related molecules such as Bad and caspase-9 [13, 14].

Ligand-dependent activation of the MET tyrosine kinase is crucial for downstream signaling that confers proliferation, cell cycle progression, migration, and motility on the cells under physiological settings. The Sema domain, which is the extracellular domain that bears structural similarity to other semaphorin family members, plays a critical role in MET activation by its ligand (see Fig. 8.1) [15–17]. Upon binding of HGF/SF with the MET Sema domain, the MET α-chain dimerizes, leading to signaling [18, 19].

Under physiological conditions, HGF/SF is secreted by fibroblasts and binds to heparan sulfate proteoglycans on cell surfaces and within the extracellular matrix [20]. The extracellular matrix often serves as a carrier of exogenous growth factors [21]. In response to inflammatory triggers, the pro-form of HGF/SF is proteolytically cleaved into an active α,β-heterodimer, stimulating the proliferation and migration of epithelial cells through MET activation [22]. It has been reported that sulfated oligosaccharides promote HGF/SF heterodimerization and govern its mitogenic activity [20], because heparin-like molecules stabilize HGF/SF oligomers, thereby facilitating MET receptor dimerization and activation.

In normal cells, the activation of MET following stimulation by HGF/SF is transient. In tumors such as breast cancer and prostate cancer, constitutive MET signaling induces an epithelial–mesenchymal transition (E–MT) (Fig. 7.1), ultimately

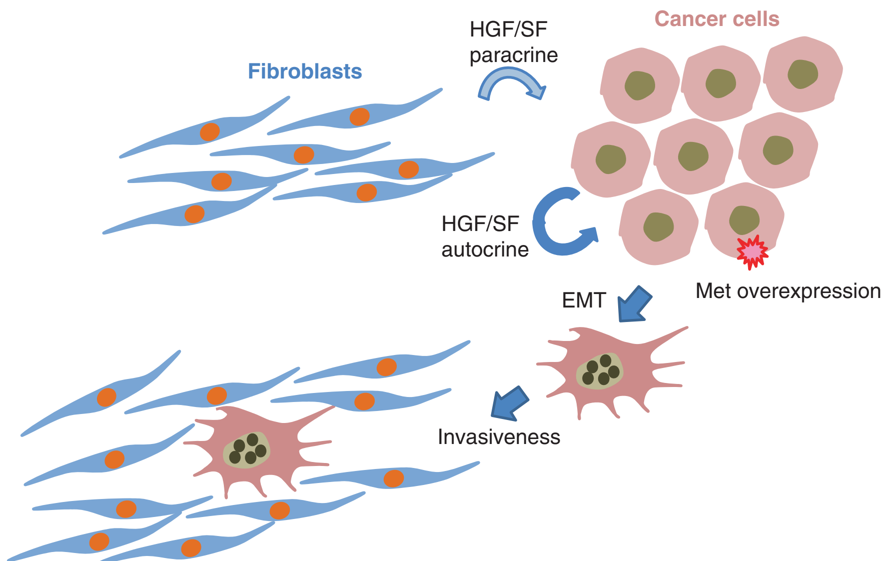

Fig. 7.1 MET signaling and cancer progression. HGF/SF-MET signaling is activated by either an HGF/SF paracrine route, an HGF/SF autocrine route, or a MET overexpression and constitutive activation, which induces an epithelial–mesenchymal transition (E–MT) and gives increased invasiveness to tumor cells

leading to carcinoma [23]. Various mechanisms can induce such MET pathway dysregulation, including ligand-dependent MET activation, *MET* mutation, *MET* amplification, and transactivation via other membrane receptors [23]. Viral or bacterial infection can also activate *MET* as an oncogene. In this chapter, we will mainly discuss the molecular basis of *MET* oncogenic activation that leads to carcinogenesis.

7.2 HGF/SF-MET Signaling Dysregulation and Cancer

7.2.1 HGF/SF-Dependent MET Activation

7.2.1.1 Paracrine HGF/SF Production by Stromal Cells

HGF/SF secreted from fibroblasts is also an important paracrine factor which induces tumor invasiveness [24]. Cancer–stroma interactions play an important role in the invasive growth of cancer cells [25]. Cross talk between invasive cancers and host stromal fibroblasts (i.e., cancer-associated fibroblasts) is strongly involved in the promotion of tumorigenesis [26], malignant cell proliferation [27], and invasion into the extracellular matrix [28], which are all enhanced by HGF/SF secretion. Factors upregulating the production of HGF/SF include IL-1, TNF [29], prostaglandins [30], and others [31]. HGF/SF and IL-6 upregulate the expression of each other's receptor, thereby promoting tumor malignancy [32]. By comparison the tumor expression studies of MET and HGF/SF were conducted with sarcoma tumor lines since MET expression in normal mesenchymal cells and tissues is low or nil, while carcinomas and normal epithelial tissues express abundant MET and HGF/SF expression.

7.2.1.2 HGF/SF-MET Autocrine Loop Activation

An HGF-MET autocrine loop significantly contributes to carcinogenesis. Experimentally, NIH/3T3 cells transformed by overexpression of human MET and HGF/SF were injected either subcutaneously or into the mammary fat pad of weanling athymic nude mice, and tumorigenicity was tested [33, 34]. Explants of tumors showed increased tumorigenicity as compared with primary transfectants. Histopathological examination revealed that these tumors were invasive. In addition, the tumor explants that showed increased levels of both HGF/SF and MET efficiently produced multifocal lung metastasis, suggesting the importance of the HGF/SF-MET autocrine signaling mechanism in tumorigenesis as well as the acquisition of metastatic potential. The most potent experimental HGF/SF and MET signaling has been observed in ligand and receptor which are co-expressed in the same cell and especially malignant when deficient in p53. Also, in a murine mammary carcinoma model, co-expression of HGF/SF and Met was proved to contribute

in part to sustained tyrosine phosphorylation of several signaling molecules such as PI3 kinase, Src, focal adhesion kinase, and phospholipase C-γ and to the growth and development of an invasive phenotype [35].

The importance of the HGF/SF-MET autocrine loop has been reported for human cancers such as colon cancer, lung adenocarcinomas, malignant mesotheliomas, and ovarian and breast cancers. Molecular co-expression of HGF/SF and MET in primary colon cancer is considered to predict a metastatic phenotype and correlates well with an advanced stage and poor survival [36]. Xenografts of NCI-H358 human lung adenocarcinoma cells having an active MET receptor showed that the autocrine loop contributed to the prominent glandular cell arrangement, functional activity, and enhanced tumorigenicity [37]. Ovarian surface epithelium from families with a history of ovarian cancer showed much higher MET expression than epithelium from families having no such history, and exogenous HGF/SF was mitogenic in the ovarian surface epithelium and was required in tumorigenic transformation [38]. This suggests that the HGF/SF-Met autocrine loop takes part in an enhanced susceptibility to ovarian carcinogenesis.

In breast cancers, a Src/Stat3-dependent mechanism is involved in regulating HGF/SF promoter activity and is linked to the transformation of mammary epithelial cells by enhancing the HGF/SF-MET autocrine loop [39]. The existence of an autocrine loop might be a useful indicator for predicting the molecular stage of cancer cells. Disrupting this loop by using targeting antibodies or decoy molecules [40, 41], small chemical molecules [42], genetic tools, etc., could be a good therapeutic strategy against cancer.

7.2.2 MET Mutation and Carcinogenesis

MET mutation has been reported to contribute to carcinogenesis or enhanced malignancy in many cancers. The first recognized cases in humans were forms of papillary renal carcinoma (PRC) in which the MET mutation activated intracellular downstream signaling [43]. The introduction of mutant MET molecules into NIH 3T3 cells formed foci in vitro, and such cells injected into nude mice were tumorigenic, which showed that MET mutation was involved in a key step of carcinogenesis. This transforming ability of PRC mutant *MET* correlated with activation of the Ras pathway [44].

7.2.2.1 *MET* Point Mutations and Hereditary Cancers

Some point mutations in the *MET* gene activate the MET tyrosine kinase, which can drive or facilitate the development of cancer. A series of reports have shown the relationship between *MET* mutation and human cancers (Table 7.1). The most common form of mutation in the MET tyrosine kinase receptor has been identified in both hereditary and sporadic forms of PRCs [43, 45–47]. Among those, most

Table 7.1 MET mutation and cancers in humans

Type of MET mutation	Organs	Cancer histology	Mechanisms of carcinogenesis	References
Missense	Kidney	Hereditary papillary renal carcinoma (HPRC); multiple, bilateral papillary renal carcinomas	Facilitate transition to the active form of the MET kinase	[43, 45–47]
Somatic mutations in the kinase domain	Liver	Childhood hepatocellular carcinomas	Acceleration of the carcinogenesis in childhood hepatocellular carcinomas not in adult carcinomas; MET mutation may have some effect on hepatitis B virus infection	[48]
Germ line juxta-membrane missense (P1009S)	Stomach	Gastric cancer	Mutated MET has increased and persistent tyrosine phosphorylation relative to the wild type in response to HGF/SF	[54]
Somatic intronic mutations that lead to an alternatively spliced transcript, a deletion of the juxta-membrane domain resulting in the loss of Cbl E3-ligase binding	Lung	Lung cancer	Decreased ubiquitination and delayed downregulation; sustained activation of phospho-MET and downstream MAPK on ligand stimulation	[55]
Somatic mutations (Y1230C and Y1235D)	Head and neck	Squamous carcinomas	The mutated MET molecules are constitutively active and confer an invasive phenotype to transfected cells	[49]
MET Y1253D-activating point mutation	Head and neck	Squamous cell carcinoma (HNSCC)	Association between MET Y1253D-activating point mutation and decreased distant metastasis- free survival in advanced HNSCC	[50]
Somatic splice site alterations at exon 14	Lung, brain	Lung adenocarcinoma, other lung neoplasms, brain glioma	Exon skipping and MET activation	[56]

mutations of the *MET* proto-oncogene were found in hereditary PRCs, whereas non-inherited PRCs showed a low frequency of *MET* mutations [45]. In hereditary PRCs, therefore, MET point mutations seem to be strongly involved in their pathogenesis. Somatic missense mutations in the kinase domain of MET molecule are known to produce childhood hepatocellular carcinomas [48]; based on the early onset of this disease, mutations of that domain of *MET* might act to accelerate the carcinogenesis.

MET-activating mutations also confer upon tumor cells invasive and metastatic properties [49, 50], resulting in poor clinical prognosis. Transgenic mice harboring mutationally activated *MET* developed metastatic mammary carcinomas in which the Ras-Raf-MEK-ERK signaling pathway was activated, enhancing cellular motility [51]. Different mutations near the signal transducer docking site of MET (Y1349 and Y1356) produce different phenotypes of transformation and invasive/metastatic activity [52]. Therefore, the main signal pathway used by a particular activating *MET* mutation is thought to be a key in determining whether the cancer cell is more proliferative or more invasive/metastatic.

A 2010 report indicated that aberrant HGF/SF-MET signaling that is often seen in human PRCs had an ability to induce centrosome amplification and chromosomal instability (CIN) via the PI3K-Akt pathway [53].

7.2.2.2 Other Types of *MET* Mutation and Cancers

While the ATP-binding site is the most important region for activating *MET* mutations [47, 48], other regions are also involved. For example, a germ line juxtamembrane missense *MET* mutation (P1009S) found in gastric cancer produces prolonged tyrosine phosphorylation in response to HGF/SF [54]. Somatic intronic mutations that lead to an alternatively spliced transcript are found in some lung cancers [55]. Such mutations cause a deletion of the juxta-membrane domain, resulting in the loss of Cbl E3-ligase binding, which ultimately delays the downregulation of MET because of decreased ubiquitination. Somatic splice-site alterations at *MET* exon 14 have been recently reported in 0.6% (221 out of 38,028) of tumor genomic profiles investigated [56].

In animal models, MET lacking the ectodomain but retaining the transmembrane and intracellular domains became an oncogenic driver [57]. Tumors developed in nude mice showed anchorage-independent growth and invasive activity.

7.2.3 MET Overexpression and Cancer Progression

The overexpression of receptor tyrosine kinases (RTKs) is the most common abnormality in human cancers. The overexpression of MET, which is observed in many cancers, leads to ligand-independent receptor dimerization and activation [15]. Non-autocrine, constitutive activation of MET is found in human anaplastic thyroid

carcinoma cells [58]. MET protein expression is reported to correlate with survival in patients with late-stage nasopharyngeal carcinoma [59]. The dimerization of MET, which is enhanced by *O*-glycosylation of MET by core 1 β1,3-galactosyltransferase, is a key event in MET activation and subsequent signal transduction inside the cells [60]. Besides dimerization, overexpression of MET itself is a unique status. Overexpressed MET in tumor cells is hyperphosphorylated and can form dimers even in the absence of its ligand, HGF/SF [16, 41], thereby activating MET signaling and leading to oncogenic transformation. Transgenic mice that overexpress MET in hepatocytes developed hepatocellular carcinoma (HCC) [61], and MET was considered to be activated by cell attachment rather than by ligand.

MET expression often correlates strongly with poor prognosis in a variety of human cancers. For example, early-stage prostate cancers are reported to be generally androgen-sensitive and either Met-negative or weakly Met-positive (if expressed). As cancer cells change from androgen-sensitive to androgen-insensitive, however, the aberrant expression of HGF/SF and MET becomes obvious. Thus, the androgen receptor (AR) negatively regulates the expression of MET [62] in a ligand-dependent manner. AR interferes with the interaction between Sp1 and the functional Sp1 binding site within the *MET* promoter. Therefore, the combination of inhibiting the HGF/SF-MET signaling pathway plus androgen ablation is considered a good option for the treatment of prostate cancers. Suppressing Met expression by gene-targeting/modifying technologies is an effective way to decrease HGF/SF-Met signaling [63].

Another example comes from the use of B16 melanoma cells for evaluating the role of MET expression in cancer malignancies [64, 65]. On the one hand, high MET expression in metastatic melanoma cells is ascribed to induction of the gene rather than preferential selection of tumor cells expressing high levels of MET [66]. On the other hand, the liver metastasis melanoma cell line B16-LS9 shows a dramatic overexpression of MET, with the gene constitutively active and more responsive to HGF/SF stimulation than in B16-F1, the parental line [67]. The relationship between MET expression and the metastatic potential of melanoma cells was further verified by testing three melanoma lines, B16-BL6, B16-F1, and B16-F10, for their metastatic potential (Fig. 7.2). The strength of MET expression was B16-F10 > B16-BL6 > B16-F1, and MET phosphorylation paralleled that order. After intravenous injection of mice with melanoma cells, the MET phosphorylation order correlated well with the lung weight (= amount of metastasized melanoma cells) as expected, and the mouse death rate also showed the same order, which shows the importance of MET activation in metastasis [68].

7.2.4 MET *Amplification and Cancers*

The relationship between amplification and carcinogenesis was first recognized when NIH 3T3 mouse fibroblasts became spontaneously transformed and showed *Met* amplification [69, 70]. Clinically, amplification of the human *MET* gene is frequent in many types of cancers, including scirrhous-type stomach cancer [71] and other types

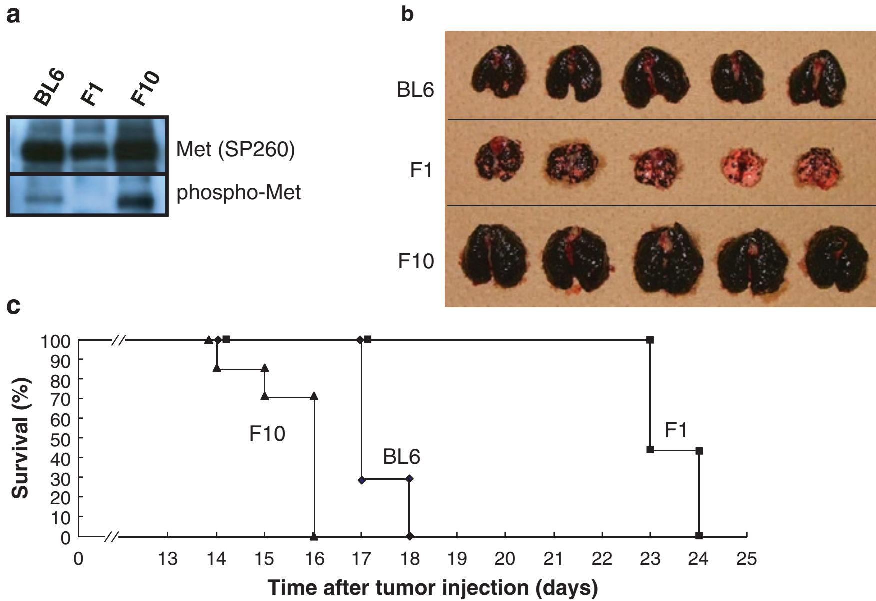

Fig. 7.2 Met expression in B16 melanoma cells and cancer malignancy. (**a**) Three B6 melanoma cell lines of common origin were used. The order of Met expression was F10 > BL6 > F1, and Met phosphorylation was the same order. (**b**) Mice were injected intravenously with 1×10^6 B6 melanoma cells of three lines (BL6, F1, F10) and were sacrificed at Day 16 . Mice injected with F10 cells showed numerous black metastatic nodules in the lungs; mice injected with F1 cells showed the lowest frequency of lung metastasis. (**c**) The duration of mouse survival after IV injection with 1×10^6 B6 melanoma cells was F1 > BL6 > F10, suggesting a close relationship between Met expression/phosphorylation and cancer malignancy

of gastric cancer [72], ovarian clear-cell adenocarcinoma [73, 74], glioblastoma [75], hepatocellular carcinoma [76], and giant cell tumor of the bone [77]. Also, a significant correlation between the amount of MET protein and an increased gene copy number has been shown in esophageal cancer [78], gastric cancer [79], and PRC [80].

MKN45 is a human gastric cancer cell line in which *MET* is amplified [81]. This line is often used for analyzing ligand-independent MET activation mechanisms (Fig. 7.3) and for the development of MET-targeting tools [82]. There is accumulating evidence about the relationship between MET expression and the aggressiveness of human carcinomas (https://resources.vai.org/Met/Index.aspx).

7.2.5 MET Transactivation Via Other Membrane Receptors

Transactivation of MET by other membrane receptors can produce cancer initiation or progression. It has been reported that integrins such as LFA-1 (lymphocyte function-associated antigen 1) and VLA4 (very late antigen 4) regulate cancer cell

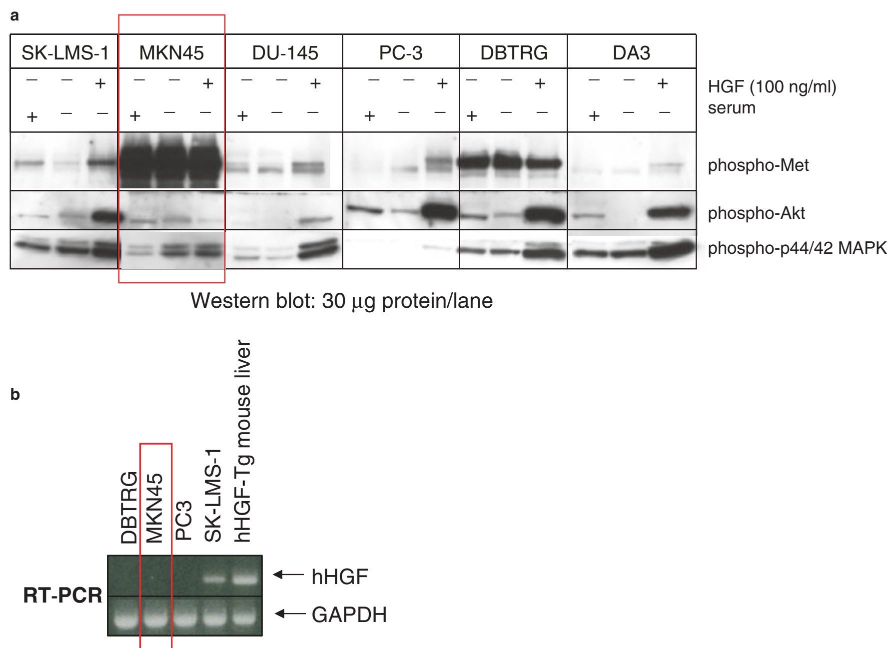

Fig. 7.3 MKN45 is a ligand-independent cell line. (**a**) In MKN45 cells from a poorly differentiated gastric cancer, Met is constitutively active, and Met phosphorylation is independent of growth factors (serum) or HGF/SF stimulation. SK-LMS-1, leiomyosarcoma; DU-145, prostate cancer; PC3, prostate cancer; DBTRG, glioblastoma; DA3, mouse mammary carcinoma. Akt and p44/42MAPK are Met downstream signals and are activated when stimulated with HGF/SF. (**b**) RT-PCR confirms that MKN45 does not produce HGF/SF. DBTRG, MKN45, and PC3 are HGF/SF-negative; SK-LMS-1 and *hHGF*-Tg mouse liver express HGS/SF

adhesion to the endothelium and the subsequent invasion into tissues. CD44 stimulates the integrin-induced adherence of colon cancer cells to the endothelial cells. CD44 stimulation also induces the expression of MET on cancer cells [83]. In this system, HGF/SF further amplifies the LFA-1-mediated adhesion of cells stimulated by CD44 signaling. In pancreatic cancer cells, α6,β4 integrin is known to upregulate several genes in the epidermal growth factor receptor (EGFR) pathway and cooperates with MET [84]. Fibronectin and vitronectin modulate the responses of endothelial cells to HGF/SF and work as an important pro-angiogenic mediator [85]. Fibronectin and vitronectin can bind to HGF/SF and form complexes that strongly promote MET–integrin association and lead to enhanced cell migration via a Ras-dependent mechanism. Extracellular matrix adhesion-dependent activation of Met is reported to be mediated by Src and the focal adhesion kinase (FAK) signaling pathway during transformation of breast epithelial cells [86].

Cell–matrix adhesion of the cancer cells is also reported to be correlated with constitutive activation of *MET* [87]. Fibronectin is a unique molecule for the invasive and metastatic capacity of ovarian cancers [88], and through an α5,β1-integrin/

MET/FAK/Src-dependent signaling pathway, Met downstream signaling is upregulated in an HGF/SF-independent manner. Also, cellular adherence is proved to be an important event in eliciting ligand-independent activation of MET [89]. The tyrosine phosphorylation of Met in mouse melanoma cells was compared before and after attachment to substrata, and the results showed the involvement of mechanical stimuli but not biochemical stimuli. This ligand-independent activation of Met occurred in several varieties of tumor cells but not in normal endothelial cells.

Because co-activation of MET and EGFR mediated by cross talk between these two molecules is thought to be involved in cancer progression, blocking both the HGF/SF-Met and EGFR signaling cascades for cancer treatment may be a good strategy for overcoming cross talk-related resistance to EGFR inhibitors [90, 91]. From this viewpoint, genomic profiling to see whether other genes are amplified in MET-activated tumors is effective in predicting the effectiveness of molecular targeting drugs [92]. Treating lung cancer with an EGFR inhibitor often induces resistant tumors which have MET amplification [93], suggesting that MET amplification could also be involved in resistance to other RTK inhibitors. The cross talk between MET and other signaling pathways and its implications for therapeutics are discussed in Chap. 8 [91].

7.2.6 Infectious Disease-Mediated Activation of the MET Pathway in Cancer

Many pathogens are thought to use the host HGF/SF-MET system to establish a comfortable environment for infection [94]. The inflammatory process caused by infection, in combination with the effect of viral/bacterial proteins, induces an HGF/SF-dependent MET activation and pushes the cell cycle into S phase. MET activation and its subsequent biological effects are often mediated by an autocrine HGF/SF circuit; for example, in viral-related carcinogenesis of human malignant mesothelioma [95]. In gastric cancers, EBV infection is reported to be associated with abnormal MET expression [96].

The agent that is closely involved in gastric cancers is *Helicobacter pylori*. The *H. pylori* virulence factor CagA associates with MET, activates intracellular signaling, and induces the proliferation of gastric epithelial cells [97]. *H. pylori* stimulates the Wnt/β-catenin pathway by activating MET and EGFR [98], which presumably play a key role in the development of gastric cancers. The HGF/SF-MET pathway has also been suggested to contribute to lymphomagenesis in MALT (mucosa-associated lymphoid tissue) lymphoma after *H. heilmannii* infection [99]. There is also accumulating evidence that hepatocellular carcinomas caused by hepatitis B virus (HBV) or hepatitis C virus (HCV) infection are often associated with increased amounts of HGF/SF or increased MET activation [100, 101].

7.2.6.1 *Helicobacter pylori* and MET Stimulation in Gastric Cancer

H. pylori infection is considered to be involved in the carcinogenesis of gastric cancers by interacting with gastric epithelial cells and activating important oncogenic signaling pathways. Recently we have shown that high MET expression is closely related to a poor prognosis of gastric cancers with *H. pylori* infection, but this is not the case without *H. pylori* infection [102]. The activity of *H. pylori* in the growth of gastric cancers is ascribed to its lipopolysaccharide, which stimulates the Toll-like receptor 4 pathway in cancer cells, causing proliferation, and attenuates the antitumor activities of human mononuclear cells [103].

The relation of *H. pylori* to MET activation was recognized when it was found that the *H. pylori* effector protein CagA [104] targets MET and promotes cellular processes leading to a forceful motogenic response. Via a type IV secretion system, CagA is translocated into epithelial cells and modulates intracellular MET activity [105]. Gastric epithelial cell invasion after CagA stimulation is mediated through a MET-dependent signaling pathway and an increase in MMP-2 and MMP-9 activity [106]. The CRPIA motif in non-phosphorylated CagA is thought to interact with activated MET, which leads to sustained activation of the PI3K/Akt pathway and ultimately to the activation of β-catenin and NF-κB signaling [107]. CagA is also involved in *H. pylori*-induced loss of gastric epithelial cell adhesion [108].

7.2.6.2 Hepatitis Viruses and MET Activation in Liver Cancer

The MET activation pathway is considered one of the most important pathways closely involved in hepatocarcinogenesis [109]. Significant increases in HGF/SF and EGF in patients with active HBV infection have been reported. The activation of those liver-regeneration factors may be a risk factor for establishing viral persistence [110], thus contributing to the progression of chronic disease and ultimately to hepatocellular carcinoma (HCC). Serum HGF/SF in patients with chronic hepatitis B was significantly correlated with serum alanine aminotransferase (ALT) and HBV DNA [111], suggesting that HGF/SF promotes viral replication and is involved in the destruction of hepatocytes. In a model of HBV-associated HCC, HGF/SF produced by cells in the inflammatory and cirrhotic lesions of a precancerous liver plays a key role in hepatic oncogenesis by stimulating the production of liver regeneration nodules [112]. In the analysis of human HCC samples, the processed form of p145 β-MET was significantly greater in tumor tissue than in non-tumor areas [113]. This also suggests that processing of the MET pro-receptor is closely associated with regeneration and carcinogenesis of the liver.

The pathogenesis of liver tumors in mice expressing conditional transgenes of *MET* in their hepatocytes has been studied [114]; the genotypes of the resulting hyperplasia and benign and malignant tumors resembled those of the human counterparts. This strongly supports an indispensable role for MET in the genesis of human liver tumors caused by HBV and HCV infection, because hepatitis induces the cycles of hepatocyte destruction and regeneration and HGF/SF-MET signaling

is thought to be strongly involved in those steps. Another study using *MET* transgenic (Tg) mice showed that the prognostic significance of gene expression signatures between mouse models and human samples was parallel and could be used as biomarkers for HCC. Especially, mouse liver tumors were most similar to a subset of patient samples characterized by activation of the Wnt pathway [100]. Mouse models showing overexpression of HGF/SF have been reported to strongly promote HBV-induced HCC progression [101]. The analysis of molecular signatures showed that the patterns were similar to human HCC cases, with overall shorter survival in both Myc/TGF-α-Tg and HGF/SF-Tg animals, suggesting the importance of these genes in HBV/HCV-induced HCC.

7.3 Carcinogenesis and Mouse Models Targeting MET

Genetically modified mouse models have been powerful tools for studying the roles of HGF and MET in cancer initiation and progression. In knock-in animals, the *HGF* or *MET* genes are replaced with a mutated functional gene. Since the inserted mutant gene is located exactly in the same place as the original and its expression is controlled under the original promoter, the natural course of the effect of mutant gene can be investigated. *MET* knockout models were produced for the analysis of its biological function during embryogenesis. Because knocking out *MET* causes embryonic death, conditional knockouts are used to eliminate *MET* expression in adult mice. Human *HGF* transgenic mouse models are used to study the role of HGF/SF-paracrine-dependent tumor growth and for preclinical evaluation of MET-targeted therapeutics.

7.3.1 MET Knock-in Mouse Models and Carcinogenesis

Since MET-activating mutations were identified in human carcinomas, experimental approaches to clarify their transforming potential have been conducted both in vitro and in animal models. Experiments using NIH 3T3 cells stably transfected with murine Met^{D1246N} or Met^{M1268T} mutations revealed a direct link between Met endocytosis and tumorigenicity [115]. Those Met mutants exhibited increased endocytosis or recycling activity and decreased degradation, leading to the accumulation of Met molecules on endosomes, the activation of Rac1 GTPase, and ultimately to loss of actin stress fibers and increased cell migration. Subcutaneous grafting of the cells into nude mice showed a rapid formation of tumors.

Targeted mutations in the murine *Met* locus were used to create five knock-in (KI) mouse lines (WT, D1226N, Y1228C, M1248 T, and M1248 T/L1193 V) on a C57BL/6 J;129/SV background [116, 117]. Each mutant line developed a unique profile of tumors (Table 7.2). Sarcomas developed in Met^{D1226N}, Met^{Y1228C}, and $Met^{M1248T/L1193V}$ but not in Met^{M1248T}, whereas carcinomas developed in Met^{M1248T} but

Table 7.2 Met-activating mutations and carcinogenesis in mice

Type of Met mutation	Tumor profile			Shorter life span	Tumor incidence (%)
	Sarcomas	Carcinomas	Lymphomas		
Wild type	N	Y	Y	N	44.4
D1226N	Y	N	N	Y	83.8
Y1228C	Y	N	Y	Y	89.5
M1248 T	N	Y	Y	N	58.8
M1248 T/L1193 V (double mutation)	Y	N	Y	N	50.0

Y yes; *N* no
Data are summarized according to the report by Graveel et al. [116]

not in other lines. Lymphomas were found in most lines but not in Met^{D1226N}. Further, Met^{D1226N} and Met^{Y1228C} showed a higher incidence of tumor formation than others, suggesting they carry a higher tumorigenic potential. Glomerulonephritis and hydronephrosis were observed in some activating Met-KI mice, but no renal carcinomas were detected in those animals. Thus, activating mutations of Met were proved to be a driving force for carcinogenesis, though the effects of such mutations in mice seems to act differently from those in humans. Interestingly, nonrandom duplication of mutant *Met* alleles was observed in the Met-KI animals. This may suggest that secondary events beyond Met mutation are required for tumor progression, which has been observed in human hereditary papillary renal carcinoma cases [118, 119].

Similar animal models on different genetic backgrounds developed different tumor types. For example, murine lines with Met D1226N, M1248 T, or Y1228C on the FVB/N background developed a high incidence of mammary carcinomas with diverse histopathologies [120]. The Met$^{M1248T/L1193V}$-KI line developed the most aggressive type of mammary tumor, in which Met is highly expressed and progesterone receptor (PR) and ErbB2 are negative [121]. The tissue microarray analysis of human breast cancers confirmed the importance of high MET expression: it significantly correlated with the gene expression patterns of PR-negative/ErbB2-negative tumors and with basal breast cancers.

7.3.2 HGF/SF-Tg Mouse Models and HGF/SF-Dependent Tumor Growth

The importance of the HGF/SF-MET signaling in carcinogenesis has been proved in several HGF/SF transgenic animal models, which develop malignant melanomas [122, 123] and multifocal invasive ductal carcinomas of the mammary gland with lung metastasis [124]. Livers of HGF/SF-Tg mice exhibit a significant increase in the number of hepatocytes and in liver mass [125]. This proliferative stimulus is considered to trigger the formation of hepatocellular adenomas and/or carcinomas in most Tg mice. HGF/SF may also play a critical role in lymphangiogenesis, thereby contributing to lymphatic metastasis [126]. Mice expressing the *HGF* transgene only in

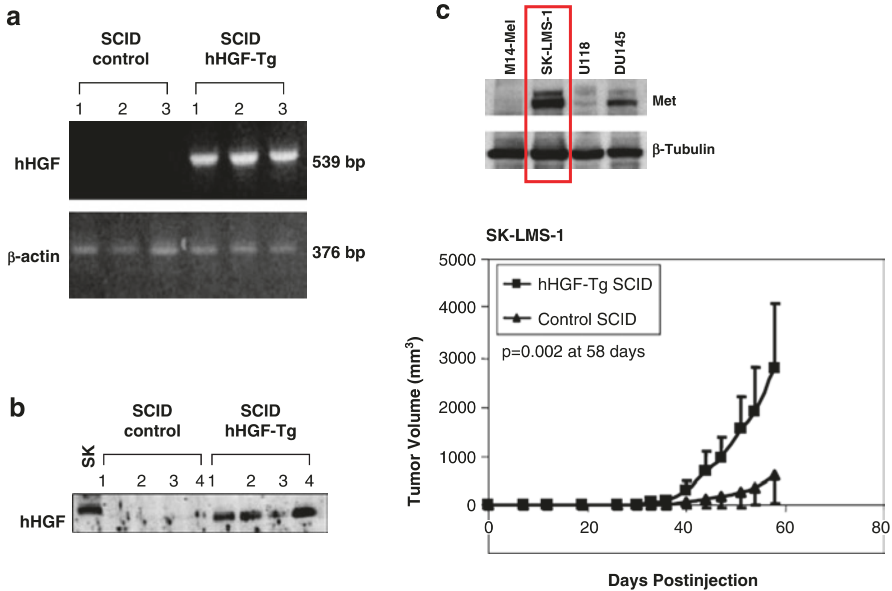

Fig. 7.4 *hHGF*-Tg SCID mouse is a good model for predicting HGF/SF-dependent tumor growth. (**a**) RT-PCR analysis showing that hHGF mRNA is produced in the liver of *hHGF*-Tg SCID mice. (**b**) Heparin bead pulldown assay showing hHGF is found in the serum of *hHGF*-Tg SCID mice. (**c**) SK-LMS-1 is a MET-positive tumor cell and grows rapidly when xenografted into *hHGF*-Tg SCID mice. Figures are from Zhang et al. [129]

the lung were used to test tobacco-induced lung carcinogenesis; the results showed that lung cancers were preferentially induced and enhanced in those mice [127].

HGF/SF-Tg mice are also used to evaluate the growth and metastatic capability of cancer cells, especially when they behave ligand dependently. Transplantation of non-autocrine melanoma cells into HGF/SF-Tg mice revealed that activation of Met was a key signal that enhanced metastatic colonization [128]. The authors generated a mouse strain transgenic for human HGF/SF on a severe combined immunodeficiency (SCID) background [129] (Fig. 7.4). Because xenogeneic tumor cells can be easily transplanted into this model and because they grow much faster than in regular SCID mice if tumor cells express Met on their surface, it is a good tool for evaluating the effectiveness of anticancer drugs and diagnostic agents.

7.3.3 *Conditional Met-KO Mouse Models and Hepatocellular Carcinoma*

Because Met knockouts are embryonic lethal [4], the indispensable role of Met in adult mice was not clear in early studies, but conditional KO technology provided new insights. Liver-specific Met-KO mice had significant impairment of liver regeneration after partial hepatectomy [130]. In that study, the activation of ERK1/2

kinase during liver regeneration depended exclusively on Met; the cell cycle halted and could not enter into S phase. In another model using a challenge with a necrogenic dose of CCl_4, Met-KO mice exhibited impaired recovery from centrolobular lesions [131]; in this case, the scattering/migration of hepatocytes into diseased areas (rather than hepatocyte proliferation) was impaired. Also, hepatocyte-specific *Met* deletion disrupted redox homeostasis, and the mice showed a hypersensitive reaction to Fas-induced liver injury [132]. Thus liver-specific Met KO models showed dysfunction of hepatocytes under stressful conditions, but those mice lived a normal life span unless they received obvious stresses to the liver [130].

A mouse model with conditional inactivation of Met in cardiomyocytes was found to be prone to cardiomyocyte hypertrophy associated with interstitial fibrosis. This shows a physiological cardioprotective role of Met in adult mice by acting as an endogenous regulator of heart function through oxidative stress control [133]. Mice with pancreatic deletion of *Met* showed significantly diminished β-cell mass, loss of regeneration, and decreased glucose tolerance, suggesting the crucial role of HGF/SF-Met signaling in pancreatic function [134]. Thus, Met function in adult mice appears to be involved in the proliferative/regenerative pathways of damaged organs.

From the oncogenic viewpoint, the partial deletion of Met, such as deletion of the ectodomain, upregulates Met phosphorylation and activates its downstream signaling pathways [57]. In an experiment of chemically induced tumor initiation, conditional Met-KO mice injected with *N*-nitrosodiethylamine showed a higher prevalence of visible liver tumors and of glutamine synthetase-positive and glucose-6-phosphatase-deficient liver lesions than did wild-type mice [135]. Glutamine synthetase is a transcriptional target of β-catenin and is therefore overexpressed in liver tumors; glucose-6-phosphatase is usually absent or lower in activity in tumor hepatocytes.

The authors have explored the role of HGF/SF-MET signaling in hepatocarcinogenesis by using genetically engineered mouse models [101]. Because transgenic mice carrying hepatitis B virus surface antigen (HBsAg-Tg mice) are reported to be a good model for developing hepatocellular carcinoma (HCC) [136], we crossed HBsAg-Tg mice with HGF/SF-Tg mice or with liver-specific Met-KO mice and monitored the incidence of HCC (Table 7.3). B6 mice with liver-specific Met-KO showed a higher incidence of HCC than B6 wild-type (WT) mice (30.0% vs. 8.3%) but a much lower incidence than B6 HBsAg-Tg mice (94.6%). There was no substantial difference in the HCC incidence between B6 HBsAg-Tg mice and those with liver-specific Met-KO (94.6% vs. 96.4%). Therefore, the effect of losing Met on the development of HCC is considered to be limited. In contrast, HGF/SF-Tg (C3H hHGF-Tg) mice showed a high HCC incidence (92.3%), and mice with both C3H hHGF-Tg and HBsAg-Tg showed 100% incidence. The C3H strain was less sensitive to HBsAg transgene-induced development of HCC than B6 (76.0% vs. 94.6%), so the effect of hHGF-Tg on hepatocarcinogenesis was remarkable. The short average survival of hHGF-Tg animals also supports the importance of constitutive/sustained activation of HGF/SF-Met signaling in the progression of HCC.

Table 7.3 Genetically engineered mouse models and HCC incidence[a]

Mouse strain	Genotype	Average survival time (weeks)	Total number of mice	Number of mice with HCC	HCC incidence (%)
B6	WT	87.1 ± 22.5	24	2	8.3
	HBsAg-Tg	73.5 ± 17.9	37	35	94.6
	Liver-specific *Met*-KO[b]	67.9 ± 30.6	20	6	30.0
	HBsAg-Tg with liver-specific *Met*-KO	70.7 ± 17.0	28	27	96.4
	HBsAg-Tg with homologous *Met* floxed alleles (but not KO)	70.9 ± 23.5	12	9	75.0
C3H	WT	91.7 ± 14.5	26	3	11.5
	HBsAg-Tg	77.4 ± 15.9	25	19	76.0
	hHGF-Tg	41.6 ± 4.7	13	12	92.3
	hHGF-Tg with *HBsAg*-Tg	49.0 ± 12.8	13	13	100

HCC hepatocellular carcinoma; *WT* wild type; *hHGF-Tg* human *HGF/SF*-transgenic; *HBsAg-Tg* hepatitis B surface antigen-transgenic

[a]Modified from the report by Xie et al. [101]

[b]Liver-specific *Met*-KO was produced by crossing *Alb-Cre/Met*$^{\text{flox/wt}}$ mice with *Met*$^{\text{flox/flox}}$ mice because *Alb-Cre/Met*$^{\text{flox/flox}}$ mice had become sterile

7.4 MET Therapeutics and Drug Resistance

Since MET became a promising anticancer target, many approaches have been developed to target it. The authors have targeted the MET molecule directly using siRNAs, which are effective against regular cancers and cancers in which MET is activated ligand independently [82]. Currently, a wide variety of small molecules that inhibit MET phosphorylation are under clinical trials [137, 138]. Other approaches against ligand-independent MET activation include a MET therapeutic protein antagonist [139] and an engineered, chemically modified antibody [140]. A list of MET inhibitors and the status of clinical trials can be found in Chap. 8.

Because activating mutations and amplification of *MET* are involved in cancer and because the behavior of cancer cells often results from an addiction to MET signaling, *MET* is an attractive candidate for targeted therapies. However, cancer cells often develop resistance to small-molecule inhibitors of MET during the treatment course. Such resistance may be partly explained by an increase of a certain type of mutated allele; for example, a M1268 T mutated allele in the time-of-progression sample relative to the pretreatment sample was found in a patient with papillary renal carcinoma [141]. Because drugs that inhibit certain mutant MET variants are being developed [142], it seems important to investigate the relationship between the type of mutation and its specific sensitivity to MET inhibitors. Also, a resistance screen using specific inhibitors is important to predict resistance mutations that could emerge during use of the inhibitor in patient treatment [143].

Changes in the gene copy number may also contribute to resistance to MET-targeting drugs. Retrospective studies in non-small-cell lung cancer have shown that a higher *MET* copy number is a negative prognostic factor, and *MET* amplification has been considered as one of the crucial events for acquired resistance in EGFR-mutated lung adenocarcinomas that are resistant to EGFR tyrosine kinase inhibitors [144]. This is because of intracellular cross talk between the MET and EGFR receptors or their signal transduction pathways [90, 145]. Thus, co-inhibition of MET and EGFR may be an effective strategy for overcoming the resistance of cancer cells to tyrosine kinase inhibitors. Further, overexpression of MET is expected to be a predictive marker for some metastatic colorectal cancer patients who might benefit from anti-EGFR therapy [146]. Because patients with MET overexpression showed less disease control and shorter progression-free survival than those with normal MET expression, the use of MET overexpression as a biomarker in combination with other markers (such as BRAF and PIK3CA mutations) would be more effective than existing methods.

7.5 Conclusion and the Way Forward

Because the activation of HGF/SF-MET signaling—whether due to MET mutation, MET overexpression, or strong activation of HGF/SF-MET autocrine/paracrine loop—plays an important role for carcinogenesis, disruption of this pathway appears as a good option for targeted therapies against specific cancers. Approaches to evaluate the molecular determinants that control MET signaling activation are being developed as biomarkers to predict the effectiveness of such therapies. For example, the monitoring of MET by IHC and FISH, of *MET* mutations, and of tissue HGF/SF should provide good data for selecting suitable drugs [147]. The presence of activating MET mutations or amplifications are key events that predict cancer malignancy and sensitivity to MET-targeting therapies [92, 101]. Resistance to MET small-molecule inhibitors [148] and unexpected side effects from some small molecules [149] have been reported, which will prompt efforts to develop more effective and less toxic drugs. From the clinical viewpoint, how can we overcome resistance to MET-targeting drugs [150] is an important issue and a long-term goal.

Acknowledgments This work was supported in part by a Grant-in-Aid for Scientific Research, Japan Society for the Promotion of Science (to N. S.; #15H04315), by the Stephen M. Coffman Charitable Trust and ETSU start-up funds (to Q. X.), and by the generosity of the Jay & Betty Van Andel Foundation (to G. V. W.). We are very grateful to David Nadziejka (Van Andel Research Institute) for technical editing of the manuscript.

References

1. Park M, Dean M, Cooper CS, Schmidt M, O'Brien SJ, Blair DG, et al. Mechanism of met oncogene activation. Cell. 1986;45:895–904.

2. Stoker M, Gherardi E, Perryman M, Gray J. Scatter factor is a fibroblast-derived modulator of epithelial cell mobility. Nature. 1987;327:239–42.
3. Nakamura T, Nishizawa T, Hagiya M, Seki T, Shimonishi M, Sugimura A, et al. Molecular cloning and expression of human hepatocyte growth factor. Nature. 1989;342:440–3.
4. Bladt F, Riethmacher D, Isenmann S, Aguzzi A, Birchmeier C. Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. Nature. 1995;376:768–71.
5. Uehara Y, Minowa O, Mori C, Shiota K, Kuno J, Noda T, et al. Placental defect and embryonic lethality in mice lacking hepatocyte growth factor/scatter factor. Nature. 1995;373:702–5.
6. Schmidt C, Bladt F, Goedecke S, Brinkmann V, Zschiesche W, Sharpe M, et al. Scatter factor/ hepatocyte growth factor is essential for liver development. Nature. 1995;373:699–702.
7. Birchmeier C, Brohmann H. Genes that control the development of migrating muscle precursor cells. Curr Opin Cell Biol. 2000;12:725–30.
8. Birchmeier C, Birchmeier W, Gherardi E, Vande Woude GF. Met, metastasis, motility and more. Nat Rev Mol Cell Biol. 2003;4:915–25.
9. Schaeper U, Gehring NH, Fuchs KP, Sachs M, Kempkes B, Birchmeier W. Coupling of Gab1 to c-Met, Grb2, and Shp2 mediates biological responses. J Cell Biol. 2000;149:1419–32.
10. Lozano E, Frasa MA, Smolarczyk K, Knaus UG, Braga VM. PAK is required for the disruption of E-cadherin adhesion by the small GTPase Rac. J Cell Sci. 2008;121:933–8.
11. Bosse T, Ehinger J, Czuchra A, Benesch S, Steffen A, Wu X, et al. Cdc42 and phosphoinositide 3-kinase drive Rac-mediated actin polymerization downstream of c-Met in distinct and common pathways. Mol Cell Biol. 2007;27:6615–28.
12. Sakkab D, Lewitzky M, Posern G, Schaeper U, Sachs M, Birchmeier W, et al. Signaling of hepatocyte growth factor/scatter factor (HGF) to the small GTPase Rap1 via the large docking protein Gab1 and the adapter protein CRKL. J Biol Chem. 2000;275:10772–8.
13. Fan S, Ma YX, Gao M, Yuan RQ, Meng Q, Goldberg ID, et al. The multisubstrate adapter Gab1 regulates hepatocyte growth factor (scatter factor)-c-Met signaling for cell survival and DNA repair. Mol Cell Biol. 2001;21:4968–84.
14. Xiao GH, Jeffers M, Bellacosa A, Mitsuuchi Y, Vande Woude GF, Testa JR. Anti-apoptotic signaling by hepatocyte growth factor/Met via the phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase pathways. Proc Natl Acad Sci U S A. 2001;98:247–52.
15. Wickramasinghe D, Kong-Beltran M. Met activation and receptor dimerization in cancer: a role for the Sema domain. Cell Cycle. 2005;4:683–5.
16. Kong-Beltran M, Stamos J, Wickramasinghe D. The Sema domain of Met is necessary for receptor dimerization and activation. Cancer Cell. 2004;6:75–84.
17. Stamos J, Lazarus RA, Yao X, Kirchhofer D, Wiesmann C. Crystal structure of the HGF betachain in complex with the Sema domain of the Met receptor. EMBO J. 2004;23:2325–35.
18. Merchant M, Ma X, Maun HR, Zheng Z, Peng J, Romero M, et al. Monovalent antibody design and mechanism of action of onartuzumab, a MET antagonist with anti-tumor activity as a therapeutic agent. Proc Natl Acad Sci U S A. 2013;110:E2987–96.
19. Tolbert WD, Daugherty J, Gao C, Xie Q, Miranti C, Gherardi E, et al. A mechanistic basis for converting a receptor tyrosine kinase agonist to an antagonist. Proc Natl Acad Sci U S A. 2007;104:14592–7.
20. Zioncheck TF, Richardson L, Liu J, Chang L, King KL, Bennett GL, et al. Sulfated oligosaccharides promote hepatocyte growth factor association and govern its mitogenic activity. J Biol Chem. 1995;270:16871–8.
21. Kanematsu A, Yamamoto S, Ozeki M, Noguchi T, Kanatani I, Ogawa O, et al. Collagenous matrices as release carriers of exogenous growth factors. Biomaterials. 2004;25:4513–20.
22. Nusrat A, Parkos CA, Bacarra AE, Godowski PJ, Delp-Archer C, Rosen EM, et al. Hepatocyte growth factor/scatter factor effects on epithelia. Regulation of intercellular junctions in transformed and nontransformed cell lines, basolateral polarization of c-met receptor in transformed and natural intestinal epithelia, and induction of rapid wound repair in a transformed model epithelium. J Clin Invest. 1994;93:2056–65.
23. Danilkovitch-Miagkova A, Zbar B. Dysregulation of Met receptor tyrosine kinase activity in invasive tumors. J Clin Invest. 2002;109:863–7.

24. Iwazawa T, Shiozaki H, Doki Y, Inoue M, Tamura S, Matsui S, et al. Primary human fibroblasts induce diverse tumor invasiveness: involvement of HGF as an important paracrine factor. Jpn J Cancer Res. 1996;87:1134–42.
25. Kataoka H, Tanaka H, Nagaike K, Uchiyama S, Itoh H. Role of cancer cell-stroma interaction in invasive growth of cancer cells. Hum Cell. 2003;16:1–14.
26. Tyan SW, Kuo WH, Huang CK, Pan CC, Shew JY, Chang KJ, et al. Breast cancer cells induce cancer-associated fibroblasts to secrete hepatocyte growth factor to enhance breast tumorigenesis. PLoS One. 2011;6:e15313.
27. Jia CC, Wang TT, Liu W, Fu BS, Hua X, Wang GY, et al. Cancer-associated fibroblasts from hepatocellular carcinoma promote malignant cell proliferation by HGF secretion. PLoS One. 2013;8:e63243.
28. Grugan KD, Miller CG, Yao Y, Michaylira CZ, Ohashi S, Klein-Szanto AJ, et al. Fibroblast-secreted hepatocyte growth factor plays a functional role in esophageal squamous cell carcinoma invasion. Proc Natl Acad Sci U S A. 2010;107:11026–31.
29. Tamura M, Arakaki N, Tsubouchi H, Takada H, Daikuhara Y. Enhancement of human hepatocyte growth factor production by interleukin-1 alpha and −1 beta and tumor necrosis factor-alpha by fibroblasts in culture. J Biol Chem. 1993;268:8140–5.
30. Ohnishi T, Suwa M, Oyama T, Arakaki N, Torii M, Daikuhara Y. Prostaglandin E2 predominantly induces production of hepatocyte growth factor/scatter factor in human dental pulp in acute inflammation. J Dent Res. 2000;79:748–55.
31. Rosen EM, Joseph A, Jin L, Rockwell S, Elias JA, Knesel J, et al. Regulation of scatter factor production via a soluble inducing factor. J Cell Biol. 1994;127:225–34.
32. To, Y, Dohi M, Matsumoto K, Tanaka R, Sato A, Nakagome K, et al. A two-way interaction between hepatocyte growth factor and interleukin-6 in tissue invasion of lung cancer cell line. Am J Respir Cell Mol Biol. 2002;27:220–6.
33. Cortner J, Vande Woude GF, Rong S. The Met-HGF/SF autocrine signaling mechanism is involved in sarcomagenesis. EXS. 1995;74:89–121.
34. Vande Woude GF, Jeffers M, Cortner J, Alvord G, Tsarfaty I, Resau J. Met-HGF/SF: tumorigenesis, invasion and metastasis. Ciba Found Symp. 1997;212:119–30; discussion 130–112, 148–154.
35. Rahimi N, Tremblay E, McAdam L, Park M, Schwall R, Elliott B. Identification of a hepatocyte growth factor autocrine loop in a murine mammary carcinoma. Cell Growth Differ. 1996;7:263–70.
36. Kammula US, Kuntz EJ, Francone TD, Zeng Z, Shia J, Landmann RG, et al. Molecular co-expression of the c-Met oncogene and hepatocyte growth factor in primary colon cancer predicts tumor stage and clinical outcome. Cancer Lett. 2007;248:219–28.
37. Yi S, Tsao MS. Activation of hepatocyte growth factor-met autocrine loop enhances tumorigenicity in a human lung adenocarcinoma cell line. Neoplasia. 2000;2:226–34.
38. Wong AS, Pelech SL, Woo MM, Yim G, Rosen B, Ehlen T, et al. Coexpression of hepatocyte growth factor-Met: an early step in ovarian carcinogenesis? Oncogene. 2001;20:1318–28.
39. Wojcik EJ, Sharifpoor S, Miller NA, Wright TG, Watering R, Tremblay EA, et al. A novel activating function of c-Src and Stat3 on HGF transcription in mammary carcinoma cells. Oncogene. 2006;25:2773–84.
40. Wright TG, Singh VK, Li JJ, Foley JH, Miller F, Jia Z, et al. Increased production and secretion of HGF alpha-chain and an antagonistic HGF fragment in a human breast cancer progression model. Int J Cancer. 2009;125:1004–15.
41. Michieli P, Mazzone M, Basilico C, Cavassa S, Sottile A, Naldini L, et al. Targeting the tumor and its microenvironment by a dual-function decoy Met receptor. Cancer Cell. 2004;6:61–73.
42. Mukohara T, Civiello G, Davis IJ, Taffaro ML, Christensen J, Fisher DE, et al. Inhibition of the met receptor in mesothelioma. Clin Cancer Res. 2005;11:8122–30.
43. Jeffers M, Schmidt L, Nakaigawa N, Webb CP, Weirich G, Kishida T, et al. Activating mutations for the met tyrosine kinase receptor in human cancer. Proc Natl Acad Sci U S A. 1997;94:11445–50.
44. Giordano S, Maffe A, Williams TA, Artigiani S, Gual P, Bardelli A, et al. Different point mutations in the met oncogene elicit distinct biological properties. FASEB J. 2000;14:399–406.

45. Schmidt L, Junker K, Nakaigawa N, Kinjerski T, Weirich G, Miller M, et al. Novel mutations of the MET proto-oncogene in papillary renal carcinomas. Oncogene. 1999;18:2343–50.
46. Schmidt L, Duh FM, Chen F, Kishida T, Glenn G, Choyke P, et al. Germline and somatic mutations in the tyrosine kinase domain of the MET proto-oncogene in papillary renal carcinomas. Nat Genet. 1997;16:68–73.
47. Olivero M, Valente G, Bardelli A, Longati P, Ferrero N, Cracco C, et al. Novel mutation in the ATP-binding site of the MET oncogene tyrosine kinase in a HPRCC family. Int J Cancer. 1999;82:640–3.
48. Park WS, Dong SM, Kim SY, Na EY, Shin MS, Pi JH, et al. Somatic mutations in the kinase domain of the Met/hepatocyte growth factor receptor gene in childhood hepatocellular carcinomas. Cancer Res. 1999;59:307–10.
49. Di Renzo MF, Olivero M, Martone T, Maffe A, Maggiora P, Stefani AD, et al. Somatic mutations of the MET oncogene are selected during metastatic spread of human HNSC carcinomas. Oncogene. 2000;19:1547–55.
50. Ghadjar P, Blank-Liss W, Simcock M, Hegyi I, Beer KT, Moch H, et al. MET Y1253D-activating point mutation and development of distant metastasis in advanced head and neck cancers. Clin Exp Metastasis. 2009;26:809–15.
51. Jeffers M, Fiscella M, Webb CP, Anver M, Koochekpour S, Vande Woude GF. The mutationally activated Met receptor mediates motility and metastasis. Proc Natl Acad Sci U S A. 1998;95:14417–22.
52. Giordano S, Bardelli A, Zhen Z, Menard S, Ponzetto C, Comoglio PM. A point mutation in the MET oncogene abrogates metastasis without affecting transformation. Proc Natl Acad Sci U S A. 1997;94:13868–72.
53. Nam HJ, Chae S, Jang SH, Cho H, Lee JH. The PI3K-Akt mediates oncogenic Met-induced centrosome amplification and chromosome instability. Carcinogenesis. 2010;31:1531–40.
54. Lee JH, Han SU, Cho H, Jennings B, Gerrard B, Dean M, et al. A novel germ line juxtamembrane Met mutation in human gastric cancer. Oncogene. 2000;19:4947–53.
55. Kong-Beltran M, Seshagiri S, Zha J, Zhu W, Bhawe K, Mendoza N, et al. Somatic mutations lead to an oncogenic deletion of met in lung cancer. Cancer Res. 2006;66:283–9.
56. Frampton GM, Ali SM, Rosenzweig M, Chmielecki J, Lu X, Bauer TM, et al. Activation of MET via diverse exon 14 splicing alterations occurs in multiple tumor types and confers clinical sensitivity to MET inhibitors. Cancer Discov. 2015;5:850–9.
57. Merlin S, Pietronave S, Locarno D, Valente G, Follenzi A, Prat M. Deletion of the ectodomain unleashes the transforming, invasive, and tumorigenic potential of the MET oncogene. Cancer Sci. 2009;100:633–8.
58. Bergstrom JD, Hermansson A, Diaz de Stahl T, Heldin NE. Non-autocrine, constitutive activation of Met in human anaplastic thyroid carcinoma cells in culture. Br J Cancer. 1999;80:650–6.
59. Qian CN, Guo X, Cao B, Kort EJ, Lee CC, Chen J, et al. Met protein expression level correlates with survival in patients with late-stage nasopharyngeal carcinoma. Cancer Res. 2002;62:589–96.
60. Wu YM, Liu CH, Huang MJ, Lai HS, Lee PH, Hu RH, et al. C1GALT1 enhances proliferation of hepatocellular carcinoma cells via modulating MET glycosylation and dimerization. Cancer Res. 2013;73:5580–90.
61. Wang R, Ferrell LD, Faouzi S, Maher JJ, Bishop JM. Activation of the Met receptor by cell attachment induces and sustains hepatocellular carcinomas in transgenic mice. J Cell Biol. 2001;153:1023–34.
62. Verras M, Lee J, Xue H, Li TH, Wang Y, Sun Z. The androgen receptor negatively regulates the expression of c-Met: implications for a novel mechanism of prostate cancer progression. Cancer Res. 2007;67:967–75.
63. Shinomiya N, Vande Woude GF. Suppression of met expression: a possible cancer treatment. Commentary re: S. J. Kim et al., reduced c-Met expression by an adenovirus expressing a c-Met ribozyme inhibits tumorigenic growth and lymph node metastases of PC3-LN4 prostate tumor cells in an orthotopic nude mouse model. Clin Cancer Res. 14:5161-5170, 2003. Clin Cancer Res. 2003;9:5085–90.

64. Rusciano D, Lorenzoni P, Burger M. Murine models of liver metastasis. Invasion Metastasis. 1994;14:349–61.
65. Rusciano D, Lorenzoni P, Burger MM. Expression of constitutively activated hepatocyte growth factor/scatter factor receptor (c-met) in B16 melanoma cells selected for enhanced liver colonization. Oncogene. 1995;11:1979–87.
66. Imai J, Watanabe M, Sasaki M, Yamaguchi R, Tateyama S, Sugano S. Induction of c-met proto-oncogene expression at the metastatic site. Clin Exp Metastasis. 1999;17:457–62.
67. Elia G, Ren Y, Lorenzoni P, Zarnegar R, Burger MM, Rusciano D. Mechanisms regulating c-met overexpression in liver-metastatic B16-LS9 melanoma cells. J Cell Biochem. 2001;81:477–87.
68. Shinomiya N, Sakamoto N, Matsuo H, Morimoto Y, Yoshimori A, Takahasi S, et al. In silico molecular design of small molecule inhibitors for Met receptor tyrosine kinase. In: 67th Annual Meeting of the Japanese Cancer Association, Nagoya, Japan; 2008.
69. Cooper CS, Tempest PR, Beckman MP, Heldin CH, Brookes P. Amplification and overexpression of the met gene in spontaneously transformed NIH3T3 mouse fibroblasts. EMBO J. 1986;5:2623–8.
70. Hudziak RM, Lewis GD, Holmes WE, Ullrich A, Shepard HM. Selection for transformation and met protooncogene amplification in NIH 3T3 fibroblasts using tumor necrosis factor alpha. Cell Growth Differ. 1990;1:129–34.
71. Kuniyasu H, Yasui W, Kitadai Y, Yokozaki H, Ito H, Tahara E. Frequent amplification of the c-met gene in scirrhous type stomach cancer. Biochem Biophys Res Commun. 1992;189:227–32.
72. Peng Z, Zhu Y, Wang Q, Gao J, Li Y, Li Y, et al. Prognostic significance of MET amplification and expression in gastric cancer: a systematic review with meta-analysis. PLoS One. 2014;9:e84502.
73. Yamamoto S, Tsuda H, Miyai K, Takano M, Tamai S, Matsubara O. Accumulative copy number increase of MET drives tumor development and histological progression in a subset of ovarian clear-cell adenocarcinomas. Mod Pathol. 2012;25:122–30.
74. Yamashita Y, Akatsuka S, Shinjo K, Yatabe Y, Kobayashi H, Seko H, et al. Met is the most frequently amplified gene in endometriosis-associated ovarian clear cell adenocarcinoma and correlates with worsened prognosis. PLoS One. 2013;8:e57724.
75. De Bacco F, Casanova E, Medico E, Pellegatta S, Orzan F, Albano R, et al. The MET oncogene is a functional marker of a glioblastoma stem cell subtype. Cancer Res. 2012;72:4537–50.
76. Wang K, Lim HY, Shi S, Lee J, Deng S, Xie T, et al. Genomic landscape of copy number aberrations enables the identification of oncogenic drivers in hepatocellular carcinoma. Hepatology. 2013;58:706–17.
77. Saada E, Peoc'h M, Decouvelaere AV, Collard O, Peyron AC, Pedeutour F. CCND1 and MET genomic amplification during malignant transformation of a giant cell tumor of bone. J Clin Oncol. 2011;29:e86–9.
78. Miller CT, Lin L, Casper AM, Lim J, Thomas DG, Orringer MB, et al. Genomic amplification of MET with boundaries within fragile site FRA7G and upregulation of MET pathways in esophageal adenocarcinoma. Oncogene. 2006;25:409–18.
79. Lee HE, Kim MA, Lee HS, Jung EJ, Yang HK, Lee BL, et al. MET in gastric carcinomas: comparison between protein expression and gene copy number and impact on clinical outcome. Br J Cancer. 2012;107:325–33.
80. Yin X, Zhang T, Su X, Ji Y, Ye P, Fu H, et al. Relationships between chromosome 7 gain, MET gene copy number increase and MET protein overexpression in chinese papillary renal cell carcinoma patients. PLoS One. 2015;10:e0143468.
81. Fushida S, Yonemura Y, Urano T, Yamaguchi A, Miyazaki I, Nakamura T, et al. Expression of hepatocyte growth factor(hgf) and C-met gene in human gastric-cancer cell-lines. Int J Oncol. 1993;3:1067–70.
82. Shinomiya N, Gao CF, Xie Q, Gustafson M, Waters DJ, Zhang YW, et al. RNA interference reveals that ligand-independent met activity is required for tumor cell signaling and survival. Cancer Res. 2004;64:7962–70.

83. Fujisaki T, Tanaka Y, Fujii K, Mine S, Saito K, Yamada S, et al. CD44 stimulation induces integrin-mediated adhesion of colon cancer cell lines to endothelial cells by up-regulation of integrins and c-Met and activation of integrins. Cancer Res. 1999;59:4427–34.
84. Carpenter BL, Chen M, Knifley T, Davis KA, Harrison SM, Stewart RL, et al. Integrin alpha6beta4 promotes autocrine epidermal growth factor receptor (EGFR) signaling to stimulate migration and invasion toward hepatocyte growth factor (HGF). J Biol Chem. 2015;290:27228–38.
85. Rahman S, Patel Y, Murray J, Patel KV, Sumathipala R, Sobel M, et al. Novel hepatocyte growth factor (HGF) binding domains on fibronectin and vitronectin coordinate a distinct and amplified Met-integrin induced signalling pathway in endothelial cells. BMC Cell Biol. 2005;6:8.
86. Hui AY, Meens JA, Schick C, Organ SL, Qiao H, Tremblay EA, et al. Src and FAK mediate cell-matrix adhesion-dependent activation of Met during transformation of breast epithelial cells. J Cell Biochem. 2009;107:1168–81.
87. Nakamura Y, Matsubara D, Goto A, Ota S, Sachiko O, Ishikawa S, et al. Constitutive activation of c-Met is correlated with c-Met overexpression and dependent on cell-matrix adhesion in lung adenocarcinoma cell lines. Cancer Sci. 2008;99:14–22.
88. Mitra AK, Sawada K, Tiwari P, Mui K, Gwin K, Lengyel E. Ligand-independent activation of c-Met by fibronectin and alpha(5)beta(1)-integrin regulates ovarian cancer invasion and metastasis. Oncogene. 2011;30:1566–76.
89. Wang R, Kobayashi R, Bishop JM. Cellular adherence elicits ligand-independent activation of the Met cell-surface receptor. Proc Natl Acad Sci U S A. 1996;93:8425–30.
90. Karamouzis MV, Konstantinopoulos PA, Papavassiliou AG. Targeting MET as a strategy to overcome crosstalk-related resistance to EGFR inhibitors. Lancet Oncol. 2009;10:709–17.
91. Zhang YW, Staal B, Essenburg C, Lewis S, Kaufman D, Vande Woude GF. Strengthening context-dependent anticancer effects on non-small cell lung carcinoma by inhibition of both MET and EGFR. Mol Cancer Ther. 2013;12:1429–41.
92. Johnson J, Ascierto ML, Mittal S, Newsome D, Kang L, Briggs M, et al. Genomic profiling of a hepatocyte growth factor-dependent signature for MET-targeted therapy in glioblastoma. J Transl Med. 2015;13:306.
93. Ghosh G, Lian X, Kron SJ, Palecek SP. Properties of resistant cells generated from lung cancer cell lines treated with EGFR inhibitors. BMC Cancer. 2012;12:95.
94. Imamura R, Matsumoto K. Hepatocyte growth factor in physiology and infectious diseases. Cytokine. 2017;98:97–106.
95. Cacciotti P, Libener R, Betta P, Martini F, Porta C, Procopio A, et al. SV40 replication in human mesothelial cells induces HGF/Met receptor activation: a model for viral-related carcinogenesis of human malignant mesothelioma. Proc Natl Acad Sci U S A. 2001;98:12032–37.
96. Luo B, Wang Y, Wang XF, Gao Y, Huang BH, Zhao P. Correlation of Epstein-Barr virus and its encoded proteins with Helicobacter pylori and expression of c-met and c-myc in gastric carcinoma. World J Gastroenterol. 2006;12:1842–8.
97. McCracken KW, Cata EM, Crawford CM, Sinagoga KL, Schumacher M, Rockich BE, et al. Modelling human development and disease in pluripotent stem-cell-derived gastric organoids. Nature. 2014;516:400–4.
98. Song X, Xin N, Wang W, Zhao C. Wnt/beta-catenin, an oncogenic pathway targeted by H. pylori in gastric carcinogenesis. Oncotarget. 2015;6:35579–88.
99. Nakamura M, Takahashi T, Matsui H, Baniwa Y, Takahashi S, Murayama SY, et al. Alteration of angiogenesis in Helicobacter heilmannii-induced mucosa-associated lymphoid tissue lymphoma: interaction with c-Met and hepatocyte growth factor. J Gastroenterol Hepatol. 2014;29(Suppl 4):70–6.
100. Ivanovska I, Zhang C, Liu AM, Wong KF, Lee NP, Lewis P, et al. Gene signatures derived from a c-MET-driven liver cancer mouse model predict survival of patients with hepatocellular carcinoma. PLoS One. 2011;6:e24582.
101. Xie Q, Su Y, Dykema K, Johnson J, Koeman J, De Giorgi V, et al. Overexpression of HGF promotes HBV-induced hepatocellular carcinoma progression and is an effective indicator for met-targeting therapy. Genes Cancer. 2013;4:247–60.

102. Sakamoto N, Tsujimoto H, Takahata R, Cao B, Zhao P, Ito N, et al. MET4 expression predicts poor prognosis of gastric cancers with Helicobacter pylori infection. Cancer Sci. 2017;108:322–30.
103. Chochi K, Ichikura T, Kinoshita M, Majima T, Shinomiya N, Tsujimoto H, et al. Helicobacter pylori augments growth of gastric cancers via the lipopolysaccharide-toll-like receptor 4 pathway whereas its lipopolysaccharide attenuates antitumor activities of human mononuclear cells. Clin Cancer Res. 2008;14:2909–17.
104. Churin Y, Al-Ghoul L, Kepp O, Meyer TF, Birchmeier W, Naumann M. Helicobacter pylori CagA protein targets the c-Met receptor and enhances the motogenic response. J Cell Biol. 2003;161:249–55.
105. Franke R, Muller M, Wundrack N, Gilles ED, Klamt S, Kahne T, et al. Host-pathogen systems biology: logical modelling of hepatocyte growth factor and Helicobacter pylori induced c-Met signal transduction. BMC Syst Biol. 2008;2:4.
106. Oliveira MJ, Costa AC, Costa AM, Henriques L, Suriano G, Atherton JC, et al. Helicobacter pylori induces gastric epithelial cell invasion in a c-Met and type IV secretion system-dependent manner. J Biol Chem. 2006;281:34888–96.
107. Suzuki M, Mimuro H, Kiga K, Fukumatsu M, Ishijima N, Morikawa H, et al. Helicobacter pylori CagA phosphorylation-independent function in epithelial proliferation and inflammation. Cell Host Microbe. 2009;5:23–34.
108. Suzuki M, Mimuro H, Suzuki T, Park M, Yamamoto T, Sasakawa C. Interaction of CagA with Crk plays an important role in Helicobacter pylori-induced loss of gastric epithelial cell adhesion. J Exp Med. 2005;202:1235–47.
109. Bupathi M, Kaseb A, Meric-Bernstam F, Naing A. Hepatocellular carcinoma: where there is unmet need. Mol Oncol. 2015;9:1501–9.
110. Barreiros AP, Sprinzl M, Rosset S, Hohler T, Otto G, Theobald M, et al. EGF and HGF levels are increased during active HBV infection and enhance survival signaling through extracellular matrix interactions in primary human hepatocytes. Int J Cancer. 2009;124:120–9.
111. Ozden M, Kalkan A, Demirdag K, Denk A, Kilic SS. Hepatocyte growth factor (HGF) in patients with hepatitis B and meningitis. J Infect. 2004;49:229–35.
112. Rogler CE, Chisari FV. Cellular and molecular mechanisms of hepatocarcinogenesis. Semin Liver Dis. 1992;12:265–78.
113. Annen K, Nojima T, Hata Y, Uchino J, Tanaka S, Matsuda M, et al. Analysis of the hepatocyte growth factor receptor in regeneration and oncogenesis of the liver. Gen Diagn Pathol. 1996;141:179–86.
114. Tward AD, Jones KD, Yant S, Kay MA, Wang R, Bishop JM. Genomic progression in mouse models for liver tumors. Cold Spring Harb Symp Quant Biol. 2005;70:217–24.
115. Joffre C, Barrow R, Menard L, Calleja V, Hart IR, Kermorgant S. A direct role for Met endocytosis in tumorigenesis. Nat Cell Biol. 2011;13:827–37.
116. Graveel C, Su Y, Koeman J, Wang LM, Tessarollo L, Fiscella M, et al. Activating Met mutations produce unique tumor profiles in mice with selective duplication of the mutant allele. Proc Natl Acad Sci U S A. 2004;101:17198–203.
117. Graveel CR, London CA, Vande Woude GF. A mouse model of activating Met mutations. Cell Cycle. 2005;4:518–20.
118. Fischer J, Palmedo G, von Knobloch R, Bugert P, Prayer-Galetti T, Pagano F, et al. Duplication and overexpression of the mutant allele of the MET proto-oncogene in multiple hereditary papillary renal cell tumours. Oncogene. 1998;17:733–9.
119. Zhuang Z, Park WS, Pack S, Schmidt L, Vortmeyer AO, Pak E, et al. Trisomy 7-harbouring non-random duplication of the mutant MET allele in hereditary papillary renal carcinomas. Nat Genet. 1998;20:66–9.
120. Graveel CR, DeGroot JD, Sigler RE, Vande Woude GF. Germline met mutations in mice reveal mutation- and background-associated differences in tumor profiles. PLoS One. 2010;5:e13586.

121. Graveel CR, DeGroot JD, Su Y, Koeman J, Dykema K, Leung S, et al. Met induces diverse mammary carcinomas in mice and is associated with human basal breast cancer. Proc Natl Acad Sci U S A. 2009;106:12909–14.
122. Otsuka T, Takayama H, Sharp R, Celli G, LaRochelle WJ, Bottaro DP, et al. c-Met autocrine activation induces development of malignant melanoma and acquisition of the metastatic phenotype. Cancer Res. 1998;58:5157–67.
123. Noonan FP, Otsuka T, Bang S, Anver MR, Merlino G. Accelerated ultraviolet radiation-induced carcinogenesis in hepatocyte growth factor/scatter factor transgenic mice. Cancer Res. 2000;60:3738–43.
124. Gallego MI, Bierie B, Hennighausen L. Targeted expression of HGF/SF in mouse mammary epithelium leads to metastatic adenosquamous carcinomas through the activation of multiple signal transduction pathways. Oncogene. 2003;22:8498–508.
125. Sakata H, Takayama H, Sharp R, Rubin JS, Merlino G, LaRochelle WJ. Hepatocyte growth factor/scatter factor overexpression induces growth, abnormal development, and tumor formation in transgenic mouse livers. Cell Growth Differ. 1996;7:1513–23.
126. Cao R, Bjorndahl MA, Gallego MI, Chen S, Religa P, Hansen AJ, et al. Hepatocyte growth factor is a lymphangiogenic factor with an indirect mechanism of action. Blood. 2006;107:3531–6.
127. Stabile LP, Lyker JS, Land SR, Dacic S, Zamboni BA, Siegfried JM. Transgenic mice overexpressing hepatocyte growth factor in the airways show increased susceptibility to lung cancer. Carcinogenesis. 2006;27:1547–55.
128. Yu Y, Merlino G. Constitutive c-Met signaling through a nonautocrine mechanism promotes metastasis in a transgenic transplantation model. Cancer Res. 2002;62:2951–6.
129. Zhang YW, Su Y, Lanning N, Gustafson M, Shinomiya N, Zhao P, et al. Enhanced growth of human met-expressing xenografts in a new strain of immunocompromised mice transgenic for human hepatocyte growth factor/scatter factor. Oncogene. 2005;24:101–6.
130. Borowiak M, Garratt AN, Wustefeld T, Strehle M, Trautwein C, Birchmeier C. Met provides essential signals for liver regeneration. Proc Natl Acad Sci U S A. 2004;101:10608–13.
131. Huh CG, Factor VM, Sanchez A, Uchida K, Conner EA, Thorgeirsson SS. Hepatocyte growth factor/c-met signaling pathway is required for efficient liver regeneration and repair. Proc Natl Acad Sci U S A. 2004;101:4477–82.
132. Gomez-Quiroz LE, Factor VM, Kaposi-Novak P, Coulouarn C, Conner EA, Thorgeirsson SS. Hepatocyte-specific c-Met deletion disrupts redox homeostasis and sensitizes to Fas-mediated apoptosis. J Biol Chem. 2008;283:14581–9.
133. Arechederra M, Carmona R, Gonzalez-Nunez M, Gutierrez-Uzquiza A, Bragado P, Cruz-Gonzalez I, et al. Met signaling in cardiomyocytes is required for normal cardiac function in adult mice. Biochim Biophys Acta. 2013;1832:2204–15.
134. Alvarez-Perez JC, Ernst S, Demirci C, Casinelli GP, Mellado-Gil JM, Rausell-Palamos F, et al. Hepatocyte growth factor/c-Met signaling is required for beta-cell regeneration. Diabetes. 2014;63:216–23.
135. Marx-Stoelting P, Borowiak M, Knorpp T, Birchmeier C, Buchmann A, Schwarz M. Hepatocarcinogenesis in mice with a conditional knockout of the hepatocyte growth factor receptor c-Met. Int J Cancer. 2009;124:1767–72.
136. Chisari FV, Klopchin K, Moriyama T, Pasquinelli C, Dunsford HA, Sell S, et al. Molecular pathogenesis of hepatocellular carcinoma in hepatitis B virus transgenic mice. Cell. 1989;59:1145–56.
137. Cui JJ. Targeting receptor tyrosine kinase MET in cancer: small molecule inhibitors and clinical progress. J Med Chem. 2014;57:4427–53.
138. Gozdzik-Spychalska J, Szyszka-Barth K, Spychalski L, Ramlau K, Wojtowicz J, Batura-Gabryel H, et al. C-MET inhibitors in the treatment of lung cancer. Curr Treat Options in Oncol. 2014;15:670–82.

139. Olwill SA, Joffroy C, Gille H, Vigna E, Matschiner G, Allersdorfer A, et al. A highly potent and specific MET therapeutic protein antagonist with both ligand-dependent and ligand-independent activity. Mol Cancer Ther. 2013;12:2459–71.
140. Vigna E, Chiriaco C, Cignetto S, Fontani L, Basilico C, Petronzelli F, et al. Inhibition of ligand-independent constitutive activation of the Met oncogenic receptor by the engineered chemically-modified antibody DN30. Mol Oncol. 2015;9:1760–72.
141. Diamond JR, Salgia R, Varella-Garcia M, Kanteti R, LoRusso PM, Clark JW, et al. Initial clinical sensitivity and acquired resistance to MET inhibition in MET-mutated papillary renal cell carcinoma. J Clin Oncol. 2013;31:e254–8.
142. Medova M, Pochon B, Streit B, Blank-Liss W, Francica P, Stroka D, et al. The novel ATP-competitive inhibitor of the MET hepatocyte growth factor receptor EMD1214063 displays inhibitory activity against selected MET-mutated variants. Mol Cancer Ther. 2013;12:2415–24.
143. Tiedt R, Degenkolbe E, Furet P, Appleton BA, Wagner S, Schoepfer J, et al. A drug resistance screen using a selective MET inhibitor reveals a spectrum of mutations that partially overlap with activating mutations found in cancer patients. Cancer Res. 2011;71:5255–64.
144. Landi L, Minuti G, D'Incecco A, Cappuzzo F. Targeting c-MET in the battle against advanced nonsmall-cell lung cancer. Curr Opin Oncol. 2013;25:130–6.
145. Jo M, Stolz DB, Esplen JE, Dorko K, Michalopoulos GK, Strom SC. Cross-talk between epidermal growth factor receptor and c-Met signal pathways in transformed cells. J Biol Chem. 2000;275:8806–11.
146. Kishiki T, Ohnishi H, Masaki T, Ohtsuka K, Ohkura Y, Furuse J, et al. Overexpression of MET is a new predictive marker for anti-EGFR therapy in metastatic colorectal cancer with wild-type KRAS. Cancer Chemother Pharmacol. 2014;73:749–57.
147. Koeppen H, Rost S, Yauch RL. Developing biomarkers to predict benefit from HGF/MET pathway inhibitors. J Pathol. 2014;232:210–8.
148. An N, Xiong Y, LaRue AC, Kraft AS, Cen B. Activation of Pim kinases is sufficient to promote resistance to MET small-molecule inhibitors. Cancer Res. 2015;75:5318–28.
149. Infante JR, Rugg T, Gordon M, Rooney I, Rosen L, Zeh K, et al. Unexpected renal toxicity associated with SGX523, a small molecule inhibitor of MET. Investig New Drugs. 2013;31:363–9.
150. Cepero V, Sierra JR, Corso S, Ghiso E, Casorzo L, Perera T, et al. MET and KRAS gene amplification mediates acquired resistance to MET tyrosine kinase inhibitors. Cancer Res. 2010;70:7580–90.

Chapter 8
The HGF/MET Signaling and Therapeutics in Cancer

Douglas P. Thewke, Jianqun Kou, Makenzie L. Fulmer, and Qian Xie

Abstract The *Met* proto-oncogene encodes MET tyrosine kinase protein which is a receptor for hepatocyte growth factor/scatter factor (HGF/SF). HGF binds to and activates MET to regulate diversified cellular and molecular activities such as proliferation, motility, differentiation, and survival. Aberration of HGF/MET signaling plays a proven role in promoting cancer initiation and malignant progression, providing a strong rationale for targeting the MET signaling pathway in the treatment of cancer. Several anti-HGF and anti-MET monoclonal antibodies, as well as small-molecule inhibitors of MET, are being evaluated in clinical trials for the treatment of various cancers. In this chapter, we discuss the role of HGF/MET signaling in cancer development and progression, the strategies for targeting MET signaling, as well as the promises and challenges of MET-targeted therapeutics.

Keywords Hepatocyte growth factor • MET tyrosine kinase receptor • Cancer proliferation and invasion • Angiogenesis • Cancer signaling pathway • MET-targeted therapy

8.1 Introduction

Receptor tyrosine kinases (RTKs) are a family of high-affinity cell surface receptors that share a similar structural motif comprising an extracellular N-terminal ligand-binding domain, a single transmembrane-spanning domain, and an intracellular C-terminal domain with tyrosine kinase activity [1]. As receptors for a wide variety of polypeptide signals including growth factors, cytokines, and hormones, RTKs are

D.P. Thewke • J. Kou • M.L. Fulmer • Q. Xie (✉)
Department of Biomedical Sciences, Center of Excellence for Inflammation, Infectious Disease and Immunity, Quillen College of Medicine, East Tennessee State University, Johnson, TN, USA
e-mail: xieq01@etsu.edu

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N. Shinomiya et al. (eds.), *Regulation of Signal Transduction in Human Cell Research*, Current Human Cell Research and Applications,
https://doi.org/10.1007/978-981-10-7296-3_8

critical regulators of many normal and important cellular processes. Over the past two decades, substantial evidence has accumulated implicating dysregulation of RTKs and their downstream signal transduction pathways in the development and progression of multiple types of cancer [2]. The *MET* proto-oncogene product, MET, is an RTK important for modulation of signaling pathways during normal development. Aberrant MET expression and dysregulated MET signaling occur in various human malignancies and have been associated with enhanced metastatic progression and poor clinical outcomes [3, 4].

8.1.1 Structure of MET and HGF

The *MET* proto-oncogene was first identified in a human osteosarcoma cell line in 1984 [5]. It is located on chromosome 7q21–31 and is primarily expressed throughout development and adulthood on the surface of epithelial cells in various organs including the kidney, liver, prostate, pancreas, and bone marrow [6–8]. However, MET expression has also been observed in other cell types, including endothelial cells, neural cells, hepatocytes, and hematopoietic cells [9–14]. The MET receptor results from proteolytic processing of a 170 kDa precursor protein that results in a heterodimeric structure consisting of an extracelular 50 kDa α-subunit disulfide linked to a transmembrane-spanning 140 kDa β-subunit (Fig. 8.1). The extracellular region of MET contains several structural and functional domains. The N-terminal 500 residues, including the entire α-subunit and part of the β-subunit, form a semaphorin (Sema) domain, similar to those in semaphorins and plexins, which is critical for ligand binding and receptor dimerization. The Sema domain is followed by a disulfide-rich plexin-semaphorin-integrin (PSI) domain and four immunoglobulin-plexin-transcription (IPT) domains connected to a single transmembrane-spanning segment [15]. The intracellular portion of MET comprises a juxtamembrane region containing key residues (S975 and Y1003) involved in receptor downregulation and degradation, a tyrosine kinase domain containing residues that modulate catalytic activity (Y1234 and Y1235) [16], and a docking site domain in the C-terminal tail containing two tyrosine residues (Y1349 and Y1356) critical for coupling to downstream signaling pathways affecting cellular proliferation, survival, migration, invasion, and tubulogenesis [17, 18].

The ligand for MET is hepatocyte growth factor (HGF). It is a fibroblast-secreted protein that is both a potent mitogen of hepatocytes and a scatter factor, which induces motility of epithelial cells [19–21]. Similar to the maturation of MET, HGF is first produced as a precursor protein, which is proteolytically processed to a mature α/β heterodimer [22, 23] (Fig. 8.1). The HGF α-subunit has an N-terminal hairpin loop followed by four kringle-like domains that participate in protein-protein interactions. The β-subunit shares homology with serine proteases, but it has no proteolytic activity.

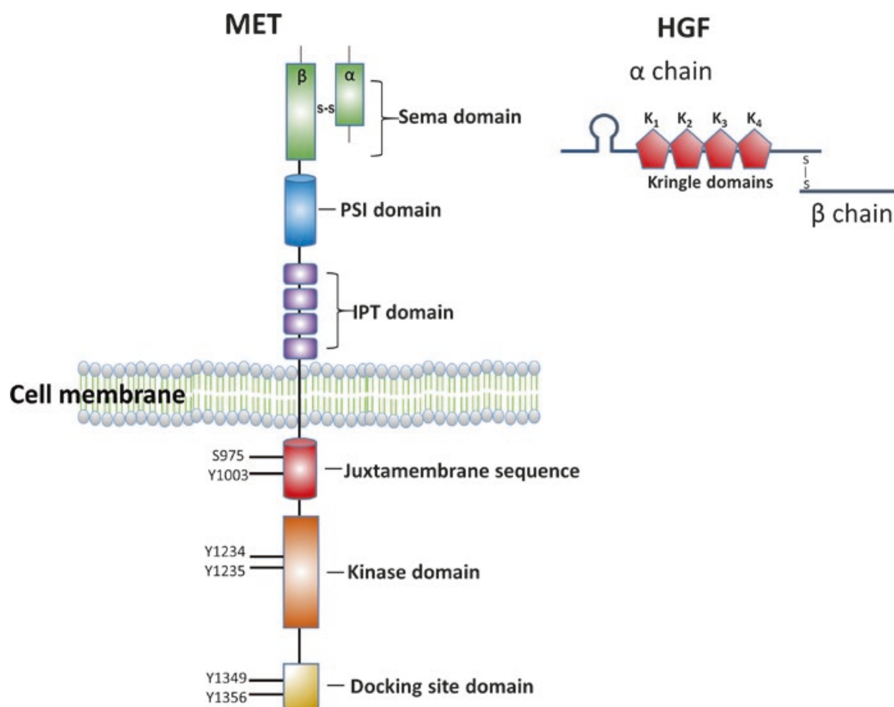

Fig. 8.1 The structure of MET and HGF. (**a**) The mature MET receptor is an α/β heterodimer that results from the posttranslational processing of a precursor into an extracellular α chain that is disulfide linked to a single-pass transmembrane β chain. The extracellular portion of MET can be divided into three separate domains: a Sema domain made up of the entire α chain and the N-terminal part of the β chain, a PSI (plexin, semaphorin, integrin) domain, and a region containing four IPT (immunoglobulin-like domain in plexins and transcription factors) domains. The intracellular portion consists of a juxtamembrane region containing residues (S975 and Y1003) involved in receptor downregulation and degradation, a tyrosine kinase domain with key regulatory residues (Y1234 and Y1235), and a C-terminal multifunctional docking site containing two tyrosines (Y1349 and Y1356) essential for coupling to adapter and effector proteins. (**b**) Mature HGF is produced by proteolytic processing of an inactive precursor which yields an active protein comprised of disulfide-linked α and β chains. The α chain contains an N-terminal hairpin loop domain followed by four kringle domains (K1-K4). The β chain has homology to serine proteases but lacks enzymatic activity

8.1.2 MET Signaling Mechanisms

MET signaling involves a complex network of multiple, discrete, and interacting signaling cascades (Fig. 8.2). The cellular effects of MET activation are likely cell type and context dependent but can include stimulation of epithelial to mesenchymal transition, proliferation, survival, migration, and tubulogenesis [24, 25]. MET signaling is activated by binding of HGF to the Sema domain followed by receptor dimerization and autophosphorylation of Y1234 and Y1235 in the kinase catalytic domain. Subsequent phosphorylation of Y1349 and Y1356 in the docking site

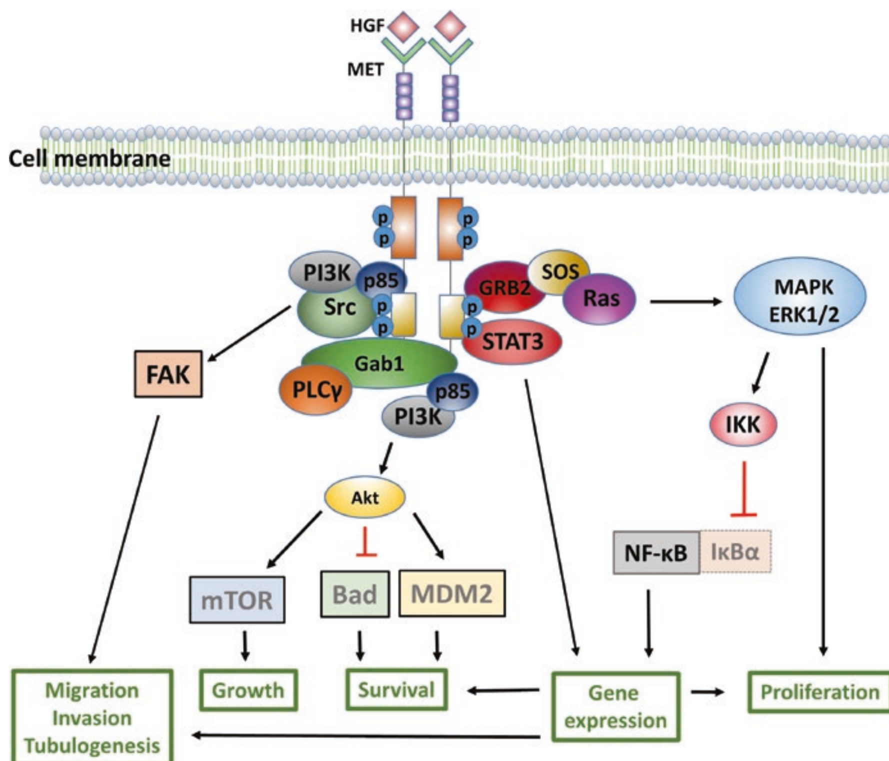

Fig. 8.2 MET signaling pathways. Binding of HGF induces MET dimerization and transphosphorylation of Y1234 and Y1235 in the kinase domain. Subsequent phosphorylation of Y1349 and Y1356 in the docking site domain creates functional recognition sites for a variety of adaptor proteins and effector kinases including growth factor receptor-bound protein 2 (Grb2), Grb2-associated adaptor protein (GAB1), SRC homology protein tyrosine phosphatase 2 (Shp2), the p85 subunit of phosphatidylinositol 3-kinase (PI3K), and signal transducer and activator of transcription 3 (STAT3). This leads to activation of downstream pathways including the mitogen-activated protein kinase (MAPK), PI3K/AKT, STAT, NFκB, and β-catenin pathways resulting in modulation of cellular proliferation, differentiation, survival, migration, invasion, and tubulogenesis

domain enables recruitment and activation of MET substrate proteins [15]. Some MET substrates are adaptor proteins such as growth factor receptor-bound protein 2 (GRB2) [26], GRB2-associated binding protein 1 (GAB1) [27], and Src homology 2-containing (SHC) [28], which couple to downstream signal transduction proteins. Of note, MET phosphorylation of GAB1 plays a crucial role in the activation of several well-studied intracellular signal transduction proteins, including phosphatidylinositol 3-kinase (PI3K), phospholipase Cγ (PLCγ), v-src sarcoma (Schmidt-Ruppin A2) viral oncogene homolog (SRC), and others [18]. Other MET substrates, such as SH2 domain-containing inositol 5-phosphatase 2 (SHIP2) [29], and signal transducer and activator of transcription 3 (STAT3) [30, 31] become activated by directly binding to the docking site domain [15].

Canonical signal transduction pathways primarily mediate the downstream effects of MET activation. The mitogen-activated protein kinase (MAPK) cascade

is stimulated by MET in the conventional fashion involving GRB2-mediated recruitment of the guanine nucleotide exchange factor SOS and the subsequent activation of RAS, Raf, MEK, and MAPK (ERK1/2). MAPK then acts on transcription factors in the nucleus to alter the expression of multiple genes involved in cellular proliferation, migration, and cell cycle progression [32, 33]. MET-mediated sequestration of the tyrosine phosphatase Src homology 2 domain-containing phosphatase 2 (SHP2) further enhances signaling through the MAPK cascade [34, 35].

The PI3K/Akt pathway is stimulated when the p85 subunit of PI3K binds to MET, either directly or indirectly by binding the adapter protein GAB1. Activation of AKT then promotes cell survival by inhibiting the pro-apoptotic activity of BCL-2 antagonist of cell death (BAD) and by activating MDM2, an E3 ubiquitin ligase that targets pro-apoptotic proteins for proteasomal degradation [36]. MET activation of AKT also stimulates protein synthesis and cellular growth by phosphorylating mammalian target of rapamycin (mTOR).

In some cell types, MET activates the expression of genes involved in proliferation, invasion, and survival by directly phosphorylating STAT3 transcription factors, which induces STAT3 dimerization and nuclear translocation [30, 31, 37]. Expression of genes involved in proliferation and tubulogenesis is stimulated by MET indirectly activating the NFκB pathway via the MAPK pathway [38]. Activated ERK1/2 phosphorylates IκB kinase (IKK) which then phosphorylates IκBα, targeting it for polyubiquitination and rapid degradation and releasing NF-κB to migrate into the nucleus to activate gene expression. MET activation of SRC family kinases activates focal adhesion kinase (FAK) that contributes to cell migration and anchorage-independent growth [39, 40]. Other signaling pathways, including the Wnt/β-catenin (see below) and the Notch/Delta [41, 42], also appear to have critical roles in MET-driven tumorigenesis.

8.1.3 Aberrant MET Signaling in Cancer

Aberrant MET signaling is significantly correlated with poor clinical outcomes in several types of cancer, including solid tumors of the lung, breast, and ovary [43–47]. A variety of mechanisms likely contribute to the activation of aberrant MET signaling in different cancers (see Chapter 7). Principals among these mechanisms are the overproduction of HGF, which results in ligand-dependent MET hyperactivity [48–51], and the overexpression of MET, which produces hypersensitivity to normal HGF levels or ligand-independent activation of signaling. Overexpression of MET primarily results from gene amplification [47, 52–57] or transcriptional activation [58–62]. Less prevalent activating mechanisms include point mutations in MET coding regions and mutations affecting alternative splicing of the primary mRNA transcript [63, 64]. Activating point mutations in MET have been found to occur in the Sema domain which controls receptor dimerization and in the juxtamembrane domain which functions in receptor downregulation [65–68]. Point mutations in the MET tyrosine kinase domain have also been noted in a few primary tumors, sometimes as a secondary response that contributes to an acquired resistance to small-molecule tyrosine kinase inhibitors [63, 69–73].

8.2 Proliferation and Invasion

A well-established hypothesis describing a phenotypic switch between tumor proliferation and invasion is epithelial-mesenchymal transition (EMT). EMT is a critical phenotypic change during embryonic development when epithelial cells lose cell-cell adhesion and transition into mesenchymal cells, which acquire the migratory property to invade into extracellular environment and participate in organ formation, such as neural crest and heart valve development and mesoderm and secondary palate formation [74–76]. In cancer progression, EMT controls the switch between cancer proliferation and metastasis (Fig. 8.3). Proliferative tumors often show an "epithelial" morphology, with tight cell junctions. Following EMT, the tumor cells become elongated and fibroblast-like; the tight cell junctions disappear. Cells degrade the extracellular matrix (ECM), producing a more invasive and migratory (mesenchymal) phenotype. The newly programmed mesenchymal cells

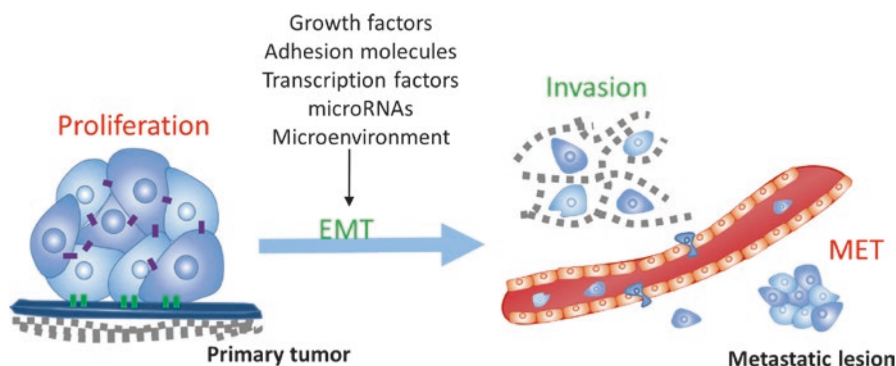

Fig. 8.3 The theory of epithelial-mesenchymal transition (EMT). During the progression of cancer, growth factors (HGF, IGF, TGFβ, Notch ligands, etc.) and other factors can initiate EMT of carcinoma cells in order to facilitate their phenotypic switches from proliferation to invasion. The EMT process is characterized by loss of epithelial markers such as E-cadherin and gain of mesenchymal markers such as β-catenin. The mesenchymal cancer cells acquire the ability to migrate into surrounding tissues and blood circulations in order to localize at secondary sites where they switch back to an epithelial phenotype (mesenchymal-epithelial transition, MET) and initiate tumor growth

migrate into blood or lymphatic circulation, where they reside until they encounter suitable extravasation sites. At such sites, a mesenchymal-epithelial transition occurs, in which the mesenchymal cells switch to the epithelial phenotype, becoming less invasive and more proliferative. With this switch, the metastatic cells grow into a secondary tumor, completing the metastasis process [75–78]. EMT is regulated by various mechanisms including growth factors, adhesion molecules, transcription factors, microRNAs, and the microenvironment, while alteration of MET signaling has been identified as playing an essential role [78–82].

8.2.1 Met Receptor Internalization

HGF is one of the classic factors initiating the EMT in cancer. Notably, in normal epithelial cells, when HGF binds to MET and activates the signaling pathway, it initiates two distinct biological processes through different substrates: by recruiting Gab1, HGF triggers downstream RAS-MAPK pathways leading to biological functions such as proliferation, invasion, and survival; by recruiting Cbl, HGF also initiates MET internalization, ubiquitination, and degradation [83]. The latter pathway serves as a preventive mechanism to avoid over activation of the MET pathway in response to HGF stimulation, which is a cause of tumorigenesis. When stimulating by HGF, the epithelial Madin-Darby canine kidney (MDCK) cells display significant phenotypic changes including loss of cell-cell adhesion, breakdown of cell junctions, and gain of cell scattering morphology indicating enhanced motility and migration. This provides a popular model system to study MET pathway regulation in EMT [68, 84–86]. Evidence showed that overexpression of mutant Cbl in MDCK cells induces the scattering phenotype even in the absence of HGF stimulation [85] and that introducing a mutant Cbl-binding domain into Met receptor provoked mesenchymal transformation leading to oncogenic activation in epithelial cells and fibroblasts [68]. These results yielded two conclusions: first, Cbl is required for MET internalization and degradation; second, loss of MET-Cbl binding causes constitutive MET activation, resulting in EMT initiation and tumorigenesis [83]. Both conclusions were recently verified by the finding in human lung tumors of a mutation within MET that leads to exon 14 skipping and a loss of the MET juxtamembrane domain containing the Cbl-binding domain [87]. More importantly, these tumor cells showed HGF-dependent tumor growth that is susceptible to MET inhibitors [88].

8.2.2 The Cadherin/Catenin Interplay

The ability of HGF to stimulate the dissociation of MDCK cell-cell attachment and change cells into a “scattering” morphology is associated with a reduced cell membrane E-cadherin expression and increased β-catenin activation [85]. All these

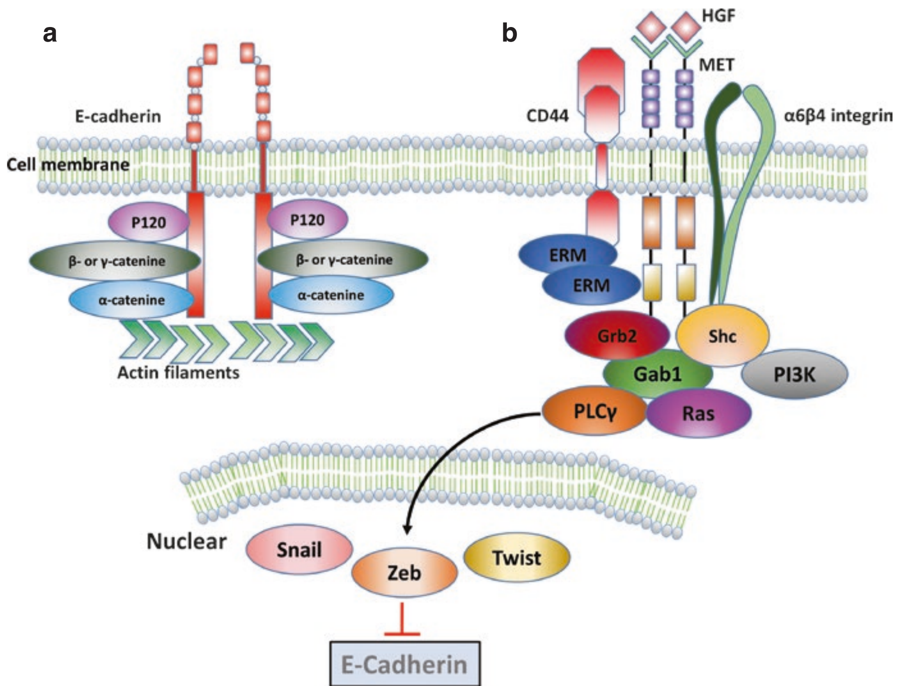

Fig. 8.4 Crosstalk between HGF and E-cadherin/β-catenin signaling. (**a**) The mature E-cadherin protein contains three distinct domains: a highly conserved cytoplasmic domain, a single-pass transmembrane domain, and an extracellular domain. The cytoplasmic tail of E-cadherin consists of the catenin-binding domain, where catenin complex forms to stabilize E-cadherin at cell-cell contact and as a major link to the actin cytoskeleton. E-cadherin forms a complex with four catenin proteins, α-catenin, β-catenin, γ-catenin, and p120 catenin [90]. (**b**) HGF binding to MET assembles a complex with CD44 and $\alpha_4\beta_6$ integrin, which facilitate signal transduction by MET, ezrin-radixin-moesin (ERM), and Shc adaptor protein, promoting tumor motility [89]. The receptor complex upregulates the expression of transcriptional repressors, such as Snail1 and Snail2, ZEB1 and ZEB2, and Twist, leading to repression of E-cadherin in nuclear

findings support the hypothesis that cadherin and catenin are important adhesion molecules in EMT [84, 89, 90] (Fig. 8.4a). Indeed, HGF promotes cancer cell invasion through loss of E-cadherin at the cell membrane and activation of β-catenin [91–93] (Fig. 8.4b). In tumors of epithelial origin, such as breast and prostate cancer, the highly proliferative tumor cells often display an “epithelial” phenotype, with tight cell junctions accompanied by overexpression of E-cadherin at the cell membrane. Loss of E-cadherin leads to tumor cell dissociation and an enhanced ability to migrate [94] and is associated with poor prognosis in human cancer patients [78, 95, 96]. As such, E-cadherin is a well-accepted biomarker of EMT in cancer. In turn, the migrating tumor cells with a “mesenchymal” phenotype are characterized by a loss of cell-cell attachment and cell membrane E-cadherin expression, with a gain of β-catenin, N-cadherin, fibronectin, and vimentin, as mesenchymal markers [97]. β-Catenin directly binds to E-cadherin intracellularly and is

a central component of the cadherin/catenin adhesive complex [98–101]. When cells display the epithelial phenotype, phosphorylated E-cadherin binds to β-catenin at the cell membrane and strengthens the cell-cell adhesion, which is essential for proliferation. Reductions in E-cadherin expression allows β-catenin to translocate into the nucleus, triggering cell-cell detachment and migration.

MET signaling can also induce EMT through HGF-independent mechanisms. Cell-cell attachment directly activates MET in cancer cells but not in normal cells [102]. Overexpression of wild-type human MET alone is sufficient to induce hepatocellular carcinoma (HCC) in mice with a high level of β-catenin found in tumors [102, 103]. Genomic analysis revealed 60% of MET-positive human HCC tumors have mutations in the β-catenin gene, *CTNNB1* [103, 104], and recent studies demonstrated that co-expression of human MET and mutant β-catenin (S33Y or S45Y) induced HCC formation in mice. Remarkably, these tumors all had active β-catenin and MET, increased glutamine synthetase and cyclin-D1 functions, upregulated MAPK/ERK and AKT/Ras/mTOR pathways, and resemble human HCC genetically and pathologically [104]. Based on these results, combination therapies targeting MET and the β-catenin pathway are under development as potential therapeutic strategies for treating malignant human HCC.

8.2.3 The Wnt/β-Catenin Pathway

In MDCK cells, the HGF-mediated loss of E-cadherin, and subsequent nuclear translocation of β-catenin, turns on Wnt-responsive genes. Moreover, co-stimulation of HGF and a Wnt pathway ligand (Wnt3) expands β-catenin activation and EMT [105]. Although under debate, studies have reported that within a heterogeneous tumor, cells that undergo EMT acquire stem cell traits and are highly tumorigenic, giving rise to the cancer stem cells (CSCs) [106, 107]. Notably, β-catenin not only serves as a critical adhesion molecule via binding to cadherins in regulating EMT (Fig. 8.4) but also is a transcription factor activated by *Wnt* signaling, a major pathway regulating normal and CSC development. Thus, crosstalk between the HGF and *Wnt* pathways overlap at the level of β-catenin in regulating EMT and CSCs [108].

Recent studies revealed that crosstalk between HGF/MET and Wnt/β-catenin regulates CSC maintenance and cancer progression in colon cancer [109, 110] (Fig. 8.5). In primary colon tumors specimens, nuclear β-catenin localization was predominantly observed in the invasive regions of colon carcinomas, supporting high Wnt/β-catenin signaling activities in mesenchymal cells [111]. Moreover, myofibroblast-secreted factors, specifically HGF, activate β-catenin transcription and subsequently CSC clonogenicity. Significantly, HGF can revert differentiated colon tumor cells back to an undifferentiated state with CSC profiles [109, 110]. As such, stromal-derived HGF maintains colon CSC property via a paracrine fashion, while the subsequent β-catenin activation promotes tumor invasive growth and malignant progression (Fig. 8.5).

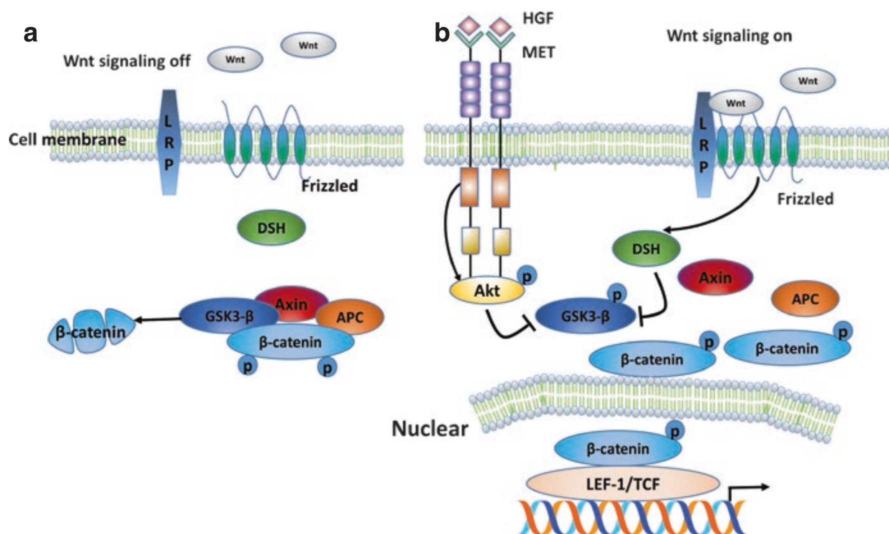

Fig. 8.5 Interactions between MET and Wnt/β-catenin signaling in colon cancer stem cells. (**a**) In the absence of Wnt ligand, cytoplasmic β-catenin binds to APC/Axin/GSK3β complex where β-catenin is phosphorylated and degraded by the proteasome. (**b**) When Wnt ligand binds to the membrane co-receptors Frizzled and LRP5 and LRP6, passing signaling through the Dishevelled (DSH) proteins, the APC/Axin/GSK3β complex is inhibited, leading to β-catenin stabilization and translocation into the nucleus where it binds to TCF/LEF, to initiate Wnt target protein expression. In colon cancer, stromal-derived HGF activates MET leading to Akt phosphorylation. Activated Akt then inhibit GSK-3β, which stabilize β-catenin followed by translocation into the nucleus [109, 110]

HGF can also activate Wnt pathways independent of β-catenin activation. Canonical Wnt signaling is initiated by the phosphorylation of the membrane co-receptors Frizzled (Fzd) and low-density lipoprotein receptor-related proteins 5 (Lrp5) and 6 (Lrp6) leading to inhibition of glycogen synthase kinase 3β (Gsk3β). Following Wnt pathway activation, β-catenin dissociates from the Gsk3β/Axin/APC complex and translocates to the nucleus, where it binds to T-cell factor/lymphoid enhancer factors (TCF/LEF) to regulate transcription of specific *Wnt* target genes (Fig. 8.5). In multiple cancer types, MET activation of Akt/NF-κB pathways upregulates the expression of LEF1, leading to tumor invasion independent of β-catenin nuclear translocation [112].

8.2.4 Transcription Factors

Transcription factors such as Snail 1, Zeb1, and Twist are traditionally implicated in promoting EMT in various systems of embryonic development and tumor progression. They also are capable of conferring stem cell-like properties, thus

strengthening the relationship between EMT and stemness [106, 113]. HGF binding to MET assembles a complex consisting of CD44 and $\alpha_4\beta_6$ integrin, leading to expression of transcriptional repressors that downregulate E-cadherin expression (Fig. 8.4b) [89]. Evidence supporting a role for HGF-mediated upregulation of Snail1 expression in the induction of EMT has been found in both MDCK and HCC cells [91, 92]. HGF also can induce Zeb1 gene expression, resulting in a loss of E-cadherin, which promotes EMT in head and neck squamous cell carcinoma [93]. In breast cancer, evidence shows that HGF induces upregulation of Twist to drive EMT [114].

8.3 Angiogenesis

Cancer progression is not determined by the tumor cells alone, but by the dynamic interaction between the tumor cells and the host tissue in which the microenvironment plays a major role in promoting or restricting tumor growth. Early studies have shown that implanting cultured tumor cells into the cornea of a rabbit eye attracted new capillaries growing from the limbus [115], while prolonged tumor dormancy was observed when tumor cells were implanted in areas lacking vasculature such as vitreous [116]. Thus, tumor cells produce soluble growth factors to initiate new vasculature from pre-existing blood vessels in order to facilitate tumor growth and metastasis, a process known as angiogenesis. During physiological angiogenesis, microenvironmental stimuli can trigger the angiogenesis cascade. For example, hypoxia can activate endothelial cells to express vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), which initiate vessel sprouting and endothelial cell proliferation. Activated endothelial cells then secrete matrix metalloproteinases (MMPs) to degrade the ECM in order to migrate and reorganize into tubular structures. In the case of cancer, the situation is more complicated, as tumor cells themselves can produce abundant growth factors leading to activation of endothelial cells. The CSCs can differentiate into endothelial cells carrying chromosomal abnormalities. In fast-growing tumors, cancer cells can form into poorly organized vasculature known as “vascular mimicry” in order to supply blood for the rapidly expanding tumor mass. Tumor growth often triggers inflammatory process as noted by the attraction of immune cells to tumors. These cells, however, also produce cytokines and proangiogenic factors such as IL-8 and IL-6, further complicating the angiogenic network [117–119].

8.3.1 The VEGF and HGF Crosstalk

VEGF, named after its biological function as a protein that stimulates vascular endothelial cell growth, is the most intensely studied angiogenic factor that initiates neovascularization. HGF is a pleiotropic cytokine regulating proliferation,

migration, and survival in many cell types including endothelial, epithelial, neuronal, and hematopoietic cells. Both VEGF and HGF can induce angiogenesis either alone or through crosstalk with each other [120, 121]. In the latter case, HGF can upregulate VEGF mRNA and protein expression in endothelial cells. Studies showed that HGF and VEGF exhibited similar effects on endothelial cell proliferation, migration, and capillary formation, and a combination of the two factors enhanced efficacy either additively or synergistically [122, 123]. Although VEGF and HGF both activate the MAPK pathway, they each utilize different signaling modulators, such as Rho or Rac. While both are members of the Rho GTPase family that regulates intracellular actin dynamics [124, 125], evidence shows that VEGF preferentially utilizes Rho to activate endothelial cell tubulogenesis, whereas HGF preferentially uses the Rac pathway [121, 126]. The Gab1 adaptor protein plays a unique role in HGF-mediated branching morphogenesis and tubule formation [127, 128]. Through a MET-binding domain that other Gab family members lack, MET can recruit Gab1 without other associated proteins such as Grb2. The direct binding of MET-Gab1 forms a more stable interaction allowing extended Gab1 phosphorylation and MAPK and AKT pathway activation, which facilitates endothelial cell tube formation.

During tumor angiogenesis, many tumor cells upregulate expression of VEGF and HGF, which sustains tumor growth via an autocrine loop and promotes endothelial cell proliferation and tubule formation via a paracrine regulation. In HCC, overexpression of HGF was observed to promote hepatocarcinogenesis through an HGF-autocrine mechanism, resulting in high levels of VEGF expression and neovascularization and demonstrating a dynamic interaction between the endothelial and tumor cells via crosstalk of the two growth factors [129]. At the molecular level, the Shc adaptor protein is essential for MET activation of VEGF expression in early tumor angiogenesis [130].

Angiogenesis is a balance of proangiogenic and antiangiogenic factors [118, 131]. Thrombospondin 1 (TSP-1) is an antiangiogenic factor that induces endothelial cells apoptosis and therefore limits vessel density in normal tissues and tumor growth [118, 132]. Remarkably, HGF upregulates VEGF and suppresses TSP-1 expression in tumor cells, leading to tumors with high vascular formation in mouse models [133]. Additionally, the HGF/MET axis upregulates urokinase activity [86, 134] and MMPs [135–137], leading to extracellular matrix (ECM) degradation that is necessary for the sprouting of new tubes.

8.3.2 The Integrin Family

Integrins are transmembrane receptors that bridge cell-cell and cell-ECM interactions and regulate the cytoskeleton, proliferation, and survival [138]. Integrins comprise heterodimeric α- and β-subunits. In mammals, there are 18 α- and 8 β-subunits forming 24 integrins binding to distinct ligands; the matching of integrins and ligands plays key roles during angiogenesis [139, 140]. In cancer, integrin signaling

also mediates tumor cell proliferation, invasion, and survival. Overexpression of integrins and ligands and crosstalk between integrins and growth factors mediate tumor angiogenesis and progression. [141–143].

HGF can activate integrins through several mechanisms and is highly associated with the β-subunit. Upon HGF stimulation, activated MET recruits integrin β4 in association with the sphingosine 1-phosphate receptor 1 (S1PR1) in endothelial cells. Formation of this complex is necessary for activating Rac signaling and maintaining vascular integrity [144, 145]. Recent studies also show that MET activation mediates recycling of integrin β1 to the plasma membrane, a crucial step in modulating endothelial cell spreading and initiation of angiogenesis. The recycling of integrin β1 requires the small GTPase Arf6 as a regulator and is independent of VEGF- or bFGF-mediated integrin activation [146, 147], therefore supporting the strategy of utilizing MET as additional antiangiogenic target to improve the moderate efficacy of VEGF inhibitors in clinical trials. HGF also binds to integrin ligands such as fibronectin and vitronectin, which amplifies the Met-integrin pathway in endothelial cells to facilitate angiogenesis [148]. The α6β4 integrin, a receptor of laminin 5, is required not only for HGF-mediated tumor cell invasion [149] but also for VEGF- or bFGF-mediated vasculature formation [150], providing an additional target for antiangiogenic therapeutics.

Integrins contributing to cell migration and angiogenesis generally require focal adhesion kinase (FAK) signaling. The N-terminal domain of FAK allows direct binding with interaction proteins such as MET, EGFR, and integrins [151]. MET activation of FAK signaling occurs either directly by binding of FAK with subsequent activation of the ERK signaling or indirectly via phosphorylation and activation of Src [152]. In lung cancer studies, direct interaction of FAK with MET is required for HGF-promoted cell invasion and the level of FAK-MET interaction is correlated with cancer cell invasiveness [153].

8.3.3 Hypoxia-Induced Tumor Angiogenesis

During cancer progression, rapid cancer cell proliferation together with vasculature abnormalities such as blood vessel occlusion or hemorrhage from areas within the solid tumors results in a hypoxic microenvironment. Tumor hypoxia is strongly associated with tumor initiation, malignant progression, and resistance to radiation and chemotherapy and is becoming an important issue in cancer treatment [154–156].

The hypoxia-inducible factors (HIFs) have principal roles in O_2 deprivation-mediated tumor progression [154, 157]. HIF-1 is a nuclear protein transcription factor composed of α and β units and functions by responding to cellular oxygen concentration. Under normoxic conditions, HIF-1α undergoes oxygen-dependent degradation in the cytosol to keep HIF-1 activity low. However, when oxygen levels decrease, HIF-1α degradation slows and HIF-1α accumulates and translocates into the nucleus, where it dimerizes with HIF-1β to form the active HIF-1 complex.

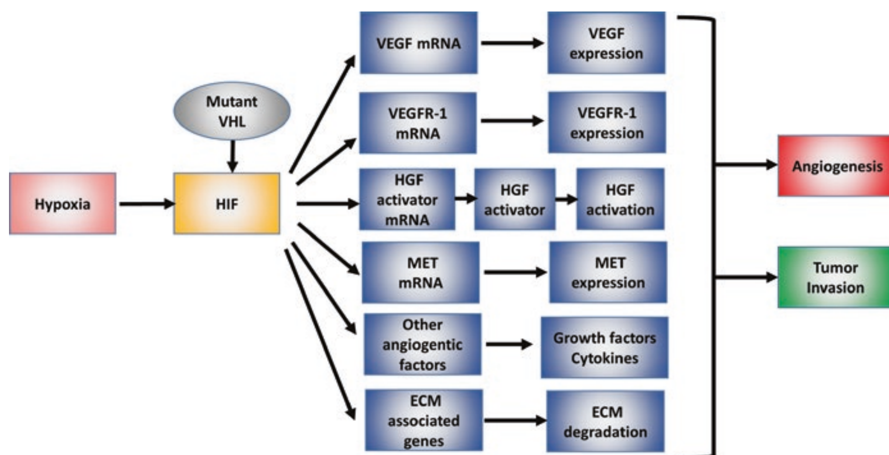

Fig. 8.6 Hypoxia-induced angiogenesis and multiple molecular pathways. Hypoxia-inducible factors (HIFs) are transcriptional factors that primarily mediate O_2 deprivation-induced angiogenesis in tumors. Hypoxia upregulates HIF-1α in endothelial and tumor cells, leading to elevated VEGF and HGF pathways, which are essential to endothelial cell survival, migration, and tube formation. Extracellular matrix degradation is also promoted to facilitate sprouting of vasculature and tumor invasion. Under normal situation, *VHL* mediates HIF-1α degradation. *VHL* mutation results in HIF-1α accumulation in the cytosol and enhances its activity

HIF-1 then binds to hypoxia-responsive elements (HREs) in the promoters of target genes to activate their expression.

The HIF pathway mediates the primary cellular response to low oxygen levels, promoting both short- and long-term adaptation to hypoxia. During cancer progression, when rapid tumor growth requires a sustained local blood supply, a long-term adaptation to hypoxia is achieved primarily through angiogenesis (Fig. 8.6). VEGF is one of the primary HIF-1 target genes [158]. In endothelial cells, HIF-1α promotes expression of VEGF and receptors VEGFR-1 and VEGF-R2, creating an autocrine VEGF signaling loop that is essential to endothelial cell survival, proliferation, migration, and tube formation. Loss of HIF-1α inhibits xenograft tumor growth due to inhibition of tumor vascularization [159, 160].

HIF-1 also regulates the HGF/MET axis and promotes invasive tumor growth [58, 161, 162]. HGF is secreted as a single-chain precursor (pro-HGF), which is cleaved by HGF activator to become a mature HGFα/β heterodimer with biological activity. Under hypoxia, HIF-1α upregulates HGF activator expression in pancreatic cancer cells, resulting in increased maturation of HGF and aberrant MET signaling [163]. HGF also protects cells from hypoxia-mediated endothelial injury and apoptosis by upregulating Bcl-2 and Bcl-xL [164, 165]. The promoter of MET contains HREs, through which HIF-1 binds and activates Met oncogene transcription. This is consistent with higher MET expression being observed in the more hypoxic regions of tumors and may result in increased responsiveness to HGF and induction of invasiveness [58]. Moreover, HIFs also regulate ECM degradation, constructing a microenvironment favorable for tumor invasion and angiogenesis [166].

While HIF-1α is the major activator of angiogenesis, mutation of the tumor suppressor gene von Hippel-Lindau (*VHL*) tremendously enhances the activity of HIF-1α, especially in renal cell carcinoma, where *VHL* mutation is frequently observed (Fig. 8.6). Because *VHL* mediates HIF-1α polyubiquitination and proteasomal degradation, inactivating mutations in *VHL* result in HIF-1α accumulation in the cytosol [154, 158, 167]. Primary renal cell carcinomas overexpress MET associated with VHL [58]. In the absence of VHL-mediated degradation, HIF-1α constitutively elevates VEGF and HGF pathways and ECM degradation, contributing to tumor angiogenesis and invasive growth.

8.4 Targeting Met Pathway in Cancer

8.4.1 Anti-HGF and Anti-MET Monoclonal Antibodies

Several different strategies have been developed to target the transformative mechanisms driven by aberrant MET signaling (Fig. 8.7). Rilotumumab (AMG-102), ficlatuzumab (AV-299), and HuL2G7 (TAK701) are monoclonal antibodies (mAbs) that bind and neutralize HGF, thereby inhibiting ligand-receptor interaction and preventing activation of downstream signaling pathways. These mAbs are under evaluation in clinical trials as either monotherapies or in combination therapies with

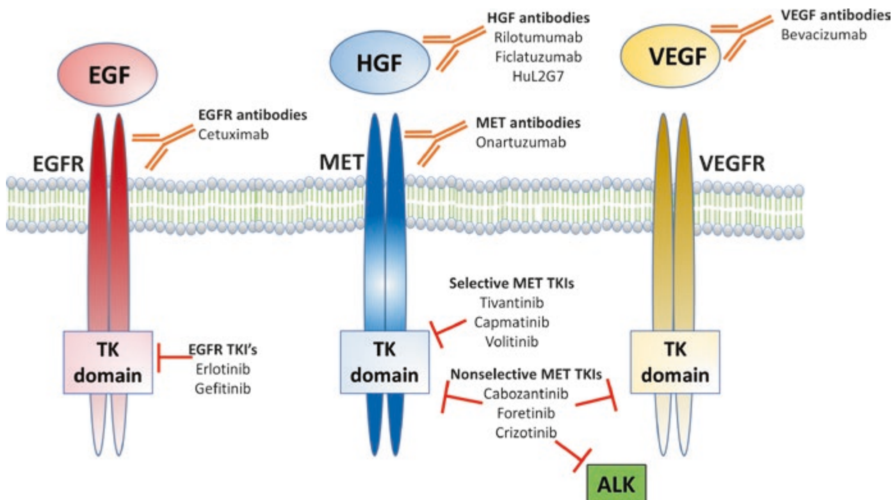

Fig. 8.7 Inhibition of MET signaling in cancer therapies. Anti-HGF and Anti-MET monoclonal antibodies interfere with HGF binding to MET, preventing MET dimerization. Selective MET tyrosine kinase inhibitors (TKIs) block MET kinase activity, while nonselective MET TKIs inhibit the kinase activity of MET and other tyrosine kinases. Combination strategies employ MET inhibitors along with inhibitors of receptors such as EGFR and VEGFR which function with MET to promote cell proliferation, survival, migration, and angiogenesis

various small-molecule inhibitors [168–173]. Similarly, mAbs targeting MET, such as onartuzumab (MetMab), CE-355621, DN-30, and LY2875358, are being developed to compete with HGF for binding to MET and to induce receptor internalization and degradation [174–177]. Of these, only MetMab has reached the clinical evaluation phase of development [178–180]. However, phase III trials failed to show clinically meaningful efficacy, and the study was halted, partially due to the lack of effective biomarkers for selecting patients [181].

8.4.2 Selective MET Tyrosine Kinase Inhibitors

Another promising strategy for blocking aberrant MET signaling is to target its intracellular tyrosine kinase activity with small-molecule tyrosine kinase inhibitors (TKIs). Tivantinib is a selective MET TKI under clinical investigation for use in therapies for some forms of lung cancers [182, 183], colon cancer [184], triple-negative breast cancer [185], and hepatocellular carcinoma [186, 187] . Several other MET-selective TKIs, such as capmatinib and volitinib, have shown efficacy in preclinical and clinical trials when used alone or in combination therapies [188–190].

8.4.3 Nonselective MET Tyrosine Kinase Inhibitors

Other TKIs under clinical investigation are nonselective and, in addition to inhibiting MET signaling, inhibit the activity of other tyrosine kinases. Crizotinib inhibits MET kinase activity as well as the activity of anaplastic lymphoma kinase (ALK) [191] and was approved by the FDA for the treatment of some late-stage lung cancers expressing ALK [192] but may also be effective in non-small cell lung cancers with aberrant MET signaling [193]. Cabozantinib inhibits MET as well as VEGFR2, AXL, RET, KIT, and FLT3 [194] and has gained FDA approval for use in the treatment of medullary thyroid cancer [195] and advanced renal cancer [196]. Cabozantinib, alone or in combination with other TKIs, is also currently under investigation for utility in the treatment of non-small cell lung cancer [197, 198], prostate cancer [199], and breast cancer [200]. Foretinib inhibits MET, VEGFR2, PDGFR, RON, FLT-1, FLT-4, and TIE2 [201, 202] and is under evaluation for the treatment of pancreatic cancer [203], renal cancer [204, 205], metastatic breast cancer [206], squamous cell carcinoma and gastric cancer [207, 208], as well as several cancers [209, 210]. Several other nonselective TKIs, including golvatinib and merestinib, are being investigated for potential anticancer activities [211–213].

8.4.4 Combination Therapies Involving MET Inhibition

The fact that the only MET inhibitors to have gained FDA approval to date are non-selective TKIs, crizotinib and cabozantinib, suggests that combination therapies involving MET inhibitors may be promising strategies for providing an overall clinical benefit. Since aberrant MET signaling has been associated with resistance to monotherapies targeting the EGFR pathway, combination therapies using mAbs and/or small-molecule TKIs to simultaneously inhibit both MET and EGFR signaling are under clinical evaluation [182–184, 214–219]. Other studies, based on the evidence linking MET signaling and angiogenesis, are investigating the efficacy of dual inhibition of MET and VEGF signaling pathways [220–223].

8.5 Summary

The success of molecular cancer therapeutics depends on the identifications of essential signaling pathways that contribute to oncogenesis and the discoveries of molecular targets that control pathway activity. Over the past 30 years, compelling evidence has shown that the HGF/MET signaling elicits important role in cell survival, proliferation, and invasion, whereas aberrant MET pathway activities promote cancer initiation and progression. With strong molecular basis, variable MET inhibitors are developed for assessing the therapeutics in human cancer [224]. As MET inhibitors are entering clinical trials, however, the therapeutic efficacy remains controversial, mainly due to a lack of patient stratification approach. It is important to develop predictive biomarkers to stratify patients vulnerable to MET therapeutics [181]. Same as other single RTK inhibitors, specific MET inhibitor can induce drug resistance due to the rapid signaling bypass; thus, combination strategies to inhibit MET signaling with other molecular pathways such as integrins, angiogenic pathways, and hypoxia pathways are to be developed to improve the therapeutic efficacy for treating malignant human cancer.

Acknowledgment We thank Dr. Robert Wondergem for critical reading. This work is supported by Stephen M. Coffman Charitable Trust and ETSU Start-up Fund (Q. X.).

References

1. Lemmon MA, Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell. 2010;141(7):1117–34.
2. Krause DS, Van Etten RA. Tyrosine kinases as targets for cancer therapy. N Engl J Med. 2005;353(2):172–87.

3. Gherardi E, et al. Targeting MET in cancer: rationale and progress. Nat Rev Cancer. 2012;12(2):89–103.
4. Trusolino L, Comoglio PM. Scatter-factor and semaphorin receptors: cell signalling for invasive growth. Nat Rev Cancer. 2002;2(4):289–300.
5. Cooper CS, et al. Molecular cloning of a new transforming gene from a chemically transformed human cell line. Nature. 1984;311(5981):29–33.
6. Comoglio PM, Giordano S, Trusolino L. Drug development of MET inhibitors: targeting oncogene addiction and expedience. Nat Rev Drug Discov. 2008;7(6):504–16.
7. Duh FM, et al. Gene structure of the human MET proto-oncogene. Oncogene. 1997;15(13):1583–6.
8. Liu Y. The human hepatocyte growth factor receptor gene: complete structural organization and promoter characterization. Gene. 1998;215(1):159–69.
9. Ding S, et al. HGF receptor up-regulation contributes to the angiogenic phenotype of human endothelial cells and promotes angiogenesis in vitro. Blood. 2003;101(12):4816–22.
10. Kajiya K, et al. Hepatocyte growth factor promotes lymphatic vessel formation and function. EMBO J. 2005;24(16):2885–95.
11. Jung W, et al. Expression and functional interaction of hepatocyte growth factor-scatter factor and its receptor c-met in mammalian brain. J Cell Biol. 1994;126(2):485–94.
12. Okano J, Shiota G, Kawasaki H. Expression of hepatocyte growth factor (HGF) and HGF receptor (c-met) proteins in liver diseases: an immunohistochemical study. Liver. 1999;19(2):151–9.
13. Kmiecik TE, et al. Hepatocyte growth factor is a synergistic factor for the growth of hematopoietic progenitor cells. Blood. 1992;80(10):2454–7.
14. Liu Y, et al. Hepatocyte growth factor and c-Met expression in pericytes: implications for atherosclerotic plaque development. J Pathol. 2007;212(1):12–9.
15. Birchmeier C, et al. Met, metastasis, motility and more. Nat Rev Mol Cell Biol. 2003;4(12):915–25.
16. Rodrigues GA, Park M. Autophosphorylation modulates the kinase activity and oncogenic potential of the Met receptor tyrosine kinase. Oncogene. 1994;9(7):2019–27.
17. Organ SL, Tsao MS. An overview of the c-MET signaling pathway. Ther Adv Med Oncol. 2011;3(1 Suppl):S7–S19.
18. Ponzetto C, et al. A multifunctional docking site mediates signaling and transformation by the hepatocyte growth factor/scatter factor receptor family. Cell. 1994;77(2):261–71.
19. Weidner KM, et al. Evidence for the identity of human scatter factor and human hepatocyte growth factor. Proc Natl Acad Sci U S A. 1991;88(16):7001–5.
20. Nakamura T, et al. Molecular cloning and expression of human hepatocyte growth factor. Nature. 1989;342(6248):440–3.
21. Stoker M, et al. Scatter factor is a fibroblast-derived modulator of epithelial cell mobility. Nature. 1987;327(6119):239–42.
22. Owen KA, et al. Pericellular activation of hepatocyte growth factor by the transmembrane serine proteases matriptase and hepsin, but not by the membrane-associated protease uPA. Biochem J. 2010;426(2):219–28.
23. Lee SL, Dickson RB, Lin CY. Activation of hepatocyte growth factor and urokinase/plasminogen activator by matriptase, an epithelial membrane serine protease. J Biol Chem. 2000;275(47):36720–5.
24. Furge KA, Zhang YW, Vande Woude GF. Met receptor tyrosine kinase: enhanced signaling through adapter proteins. Oncogene. 2000;19(49):5582–9.
25. Peruzzi B, Bottaro DP. Targeting the c-Met signaling pathway in cancer. Clin Cancer Res. 2006;12(12):3657–60.
26. Fixman ED, et al. Pathways downstream of Shc and Grb2 are required for cell transformation by the tpr-Met oncoprotein. J Biol Chem. 1996;271(22):13116–22.
27. Weidner KM, et al. Interaction between Gab1 and the c-Met receptor tyrosine kinase is responsible for epithelial morphogenesis. Nature. 1996;384(6605):173–6.

28. Pelicci G, et al. The motogenic and mitogenic responses to HGF are amplified by the Shc adaptor protein. Oncogene. 1995;10(8):1631–8.
29. Koch A, et al. The SH2-domian-containing inositol 5-phosphatase (SHIP)-2 binds to c-Met directly via tyrosine residue 1356 and involves hepatocyte growth factor (HGF)-induced lamellipodium formation, cell scattering and cell spreading. Oncogene. 2005;24(21):3436–47.
30. Zhang YW, et al. Requirement of Stat3 signaling for HGF/SF-Met mediated tumorigenesis. Oncogene. 2002;21(2):217–26.
31. Boccaccio C, et al. Induction of epithelial tubules by growth factor HGF depends on the STAT pathway. Nature. 1998;391(6664):285–8.
32. Graziani A, et al. Hepatocyte growth factor/scatter factor stimulates the Ras-guanine nucleotide exchanger. J Biol Chem. 1993;268(13):9165–8.
33. Paumelle R, et al. Hepatocyte growth factor/scatter factor activates the ETS1 transcription factor by a RAS-RAF-MEK-ERK signaling pathway. Oncogene. 2002;21(15):2309–19.
34. Maroun CR, Naujokas MA, Park M. Membrane targeting of Grb2-associated binder-1 (Gab1) scaffolding protein through Src myristoylation sequence substitutes for Gab1 pleckstrin homology domain and switches an epidermal growth factor response to an invasive morphogenic program. Mol Biol Cell. 2003;14(4):1691–708.
35. Schaeper U, et al. Coupling of Gab1 to c-Met, Grb2, and Shp2 mediates biological responses. J Cell Biol. 2000;149(7):1419–32.
36. Xiao GH, et al. Anti-apoptotic signaling by hepatocyte growth factor/Met via the phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase pathways. Proc Natl Acad Sci U S A. 2001;98(1):247–52.
37. Syed ZA, et al. HGF/c-met/Stat3 signaling during skin tumor cell invasion: indications for a positive feedback loop. BMC Cancer. 2011;11:180.
38. Müller M, Morotti A, Ponzetto C. Activation of NF-kappaB is essential for hepatocyte growth factor-mediated proliferation and tubulogenesis. Mol Cell Biol. 2002;22(4):1060–72.
39. Hui AY, et al. Src and FAK mediate cell-matrix adhesion-dependent activation of Met during transformation of breast epithelial cells. J Cell Biochem. 2009;107(6):1168–81.
40. Rahimi N, et al. c-Src kinase activity is required for hepatocyte growth factor-induced motility and anchorage-independent growth of mammary carcinoma cells. J Biol Chem. 1998;273(50):33714–21.
41. Zhang S, et al. Targeting Met and Notch in the Lfng-deficient, Met-amplified triple-negative breast cancer. Cancer Biol Ther. 2014;15(5):633–42.
42. Stella MC, et al. Negative feedback regulation of Met-dependent invasive growth by Notch. Mol Cell Biol. 2005;25(10):3982–96.
43. Shattuck DL, et al. Met receptor contributes to trastuzumab resistance of Her2-overexpressing breast cancer cells. Cancer Res. 2008;68(5):1471–7.
44. Siegfried JM, et al. Association of immunoreactive hepatocyte growth factor with poor survival in resectable non-small cell lung cancer. Cancer Res. 1997;57(3):433–9.
45. Sawada K, et al. c-Met overexpression is a prognostic factor in ovarian cancer and an effective target for inhibition of peritoneal dissemination and invasion. Cancer Res. 2007;67(4):1670–9.
46. Lo Muzio L, et al. Effect of c-Met expression on survival in head and neck squamous cell carcinoma. Tumour Biol. 2006;27(3):115–21.
47. Park S, et al. High MET copy number and MET overexpression: poor outcome in non-small cell lung cancer patients. Histol Histopathol. 2012;27(2):197–207.
48. Koochekpour S, et al. Met and hepatocyte growth factor/scatter factor expression in human gliomas. Cancer Res. 1997;57(23):5391–8.
49. Li G, et al. Downregulation of E-cadherin and Desmoglein 1 by autocrine hepatocyte growth factor during melanoma development. Oncogene. 2001;20(56):8125–35.
50. Ferracini R, et al. The Met/HGF receptor is over-expressed in human osteosarcomas and is activated by either a paracrine or an autocrine circuit. Oncogene. 1995;10(4):739–49.
51. Tuck AB, et al. Coexpression of hepatocyte growth factor and receptor (Met) in human breast carcinoma. Am J Pathol. 1996;148(1):225–32.

52. Cappuzzo F, et al. Increased MET gene copy number negatively affects survival of surgically resected non-small-cell lung cancer patients. J Clin Oncol. 2009;27(10):1667–74.
53. Okuda K, et al. Met gene copy number predicts the prognosis for completely resected non-small cell lung cancer. Cancer Sci. 2008;99(11):2280–5.
54. Ichimura E, et al. Expression of c-met/HGF receptor in human non-small cell lung carcinomas in vitro and in vivo and its prognostic significance. Jpn J Cancer Res. 1996;87(10):1063–9.
55. Takanami I, et al. Hepatocyte growth factor and c-Met/hepatocyte growth factor receptor in pulmonary adenocarcinomas: an evaluation of their expression as prognostic markers. Oncology. 1996;53(5):392–7.
56. Gumustekin M, et al. HGF/c-Met overexpressions, but not met mutation, correlates with progression of non-small cell lung cancer. Pathol Oncol Res. 2012;18(2):209–18.
57. Smolen GA, et al. Amplification of MET may identify a subset of cancers with extreme sensitivity to the selective tyrosine kinase inhibitor PHA-665752. Proc Natl Acad Sci U S A. 2006;103(7):2316–21.
58. Pennacchietti S, et al. Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. Cancer Cell. 2003;3(4):347–61.
59. Boon EM, et al. Wnt signaling regulates expression of the receptor tyrosine kinase met in colorectal cancer. Cancer Res. 2002;62(18):5126–8.
60. Kanteti R, et al. PAX5 is expressed in small-cell lung cancer and positively regulates c-Met transcription. Lab Invest. 2009;89(3):301–14.
61. Ivan M, et al. Activated ras and ret oncogenes induce over-expression of c-met (hepatocyte growth factor receptor) in human thyroid epithelial cells. Oncogene. 1997;14(20):2417–23.
62. Gambarotta G, et al. Ets up-regulates MET transcription. Oncogene. 1996;13(9):1911–7.
63. Feng Y, Thiagarajan PS, Ma PC. MET signaling: novel targeted inhibition and its clinical development in lung cancer. J Thorac Oncol. 2012;7(2):459–67.
64. Ma PC, et al. Expression and mutational analysis of MET in human solid cancers. Genes Chromosomes Cancer. 2008;47(12):1025–37.
65. Krishnaswamy S, et al. Ethnic differences and functional analysis of MET mutations in lung cancer. Clin Cancer Res. 2009;15(18):5714–23.
66. Zaffaroni D, et al. Met proto-oncogene juxtamembrane rare variations in mouse and humans: differential effects of Arg and Cys alleles on mouse lung tumorigenesis. Oncogene. 2005;24(6):1084–90.
67. Ma PC, et al. c-MET mutational analysis in small cell lung cancer: novel juxtamembrane domain mutations regulating cytoskeletal functions. Cancer Res. 2003;63(19):6272–81.
68. Peschard P, et al. Mutation of the c-Cbl TKB domain binding site on the Met receptor tyrosine kinase converts it into a transforming protein. Mol Cell. 2001;8(5):995–1004.
69. Schmidt L, et al. Germline and somatic mutations in the tyrosine kinase domain of the MET proto-oncogene in papillary renal carcinomas. Nat Genet. 1997;16(1):68–73.
70. Ma PC, et al. Functional expression and mutations of c-Met and its therapeutic inhibition with SU11274 and small interfering RNA in non-small cell lung cancer. Cancer Res. 2005;65(4):1479–88.
71. Cortesina G, et al. Molecular markers study in pTNM of squamous carcinoma of the head and neck. Acta Otorhinolaryngol Ital. 2000;20(6):380–2.
72. Walz C, Sattler M. Novel targeted therapies to overcome imatinib mesylate resistance in chronic myeloid leukemia (CML). Crit Rev Oncol Hematol. 2006;57(2):145–64.
73. Engelman JA, Jänne PA. Mechanisms of acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors in non-small cell lung cancer. Clin Cancer Res. 2008;14(10):2895–9.
74. Nieto MA. Epithelial-mesenchymal transitions in development and disease: old views and new perspectives. Int J Dev Biol. 2009;53(8–10):1541–7.
75. Thiery JP, et al. Epithelial-mesenchymal transitions in development and disease. Cell. 2009;139(5):871–90.

76. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer. 2002;2(6):442–54.
77. Birchmeier W, Birchmeier C. Epithelial-mesenchymal transitions in development and tumor progression. EXS. 1995;74:1–15.
78. De Craene B, Berx G. Regulatory networks defining EMT during cancer initiation and progression. Nat Rev Cancer. 2013;13(2):97–110.
79. Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. Nat Rev Mol Cell Biol. 2014;15(3):178–96.
80. Jung HY, Fattet L, Yang J. Molecular pathways: linking tumor microenvironment to epithelial-mesenchymal transition in metastasis. Clin Cancer Res. 2015;21(5):962–8.
81. Zhang J, Ma L. MicroRNA control of epithelial-mesenchymal transition and metastasis. Cancer Metastasis Rev. 2012;31(3–4):653–62.
82. Zhang P, Sun Y, Ma L. ZEB1: at the crossroads of epithelial-mesenchymal transition, metastasis and therapy resistance. Cell Cycle. 2015;14(4):481–7.
83. Petrelli A, et al. The endophilin-CIN85-Cbl complex mediates ligand-dependent downregulation of c-Met. Nature. 2002;416(6877):187–90.
84. Birchmeier C, Birchmeier W, Brand-Saberi B. Epithelial-mesenchymal transitions in cancer progression. Acta Anat (Basel). 1996;156(3):217–26.
85. Fournier TM, et al. Cbl-transforming variants trigger a cascade of molecular alterations that lead to epithelial mesenchymal conversion. Mol Biol Cell. 2000;11(10):3397–410.
86. Xie Q, et al. Geldanamycins exquisitely inhibit HGF/SF-mediated tumor cell invasion. Oncogene. 2005;24(23):3697–707.
87. Paik PK, et al. Response to MET inhibitors in patients with stage IV lung adenocarcinomas harboring MET mutations causing exon 14 skipping. Cancer Discov. 2015;5(8):842–9.
88. Merchant M, et al. Monovalent antibody design and mechanism of action of onartuzumab, a MET antagonist with anti-tumor activity as a therapeutic agent. Proc Natl Acad Sci U S A. 2013;110(32):E2987–96.
89. Yilmaz M, Christofori G. Mechanisms of motility in metastasizing cells. Mol Cancer Res. 2010;8(5):629–42.
90. Tian X, et al. E-cadherin/beta-catenin complex and the epithelial barrier. J Biomed Biotechnol. 2011;2011:567305.
91. Nagai T, et al. Sorafenib inhibits the hepatocyte growth factor-mediated epithelial mesenchymal transition in hepatocellular carcinoma. Mol Cancer Ther. 2011;10(1):169–77.
92. Grotegut S, et al. Hepatocyte growth factor induces cell scattering through MAPK/Egr-1-mediated upregulation of Snail. EMBO J. 2006;25(15):3534–45.
93. Susuki D, et al. Regulation of microRNA expression by hepatocyte growth factor in human head and neck squamous cell carcinoma. Cancer Sci. 2011;102(12):2164–71.
94. Serres M, et al. The disruption of adherens junctions is associated with a decrease of E-cadherin phosphorylation by protein kinase CK2. Exp Cell Res. 2000;257(2):255–64.
95. Cavallaro U, Christofori G. Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. Nat Rev Cancer. 2004;4(2):118–32.
96. Onder TT, et al. Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. Cancer Res. 2008;68(10):3645–54.
97. Zeisberg M, Neilson EG. Biomarkers for epithelial-mesenchymal transitions. J Clin Invest. 2009;119(6):1429–37.
98. Daugherty RL, Gottardi CJ. Phospho-regulation of Beta-catenin adhesion and signaling functions. Physiology (Bethesda). 2007;22:303–9.
99. Huber AH, Weis WI. The structure of the beta-catenin/E-cadherin complex and the molecular basis of diverse ligand recognition by beta-catenin. Cell. 2001;105(3):391–402.
100. Huber AH, et al. The cadherin cytoplasmic domain is unstructured in the absence of beta-catenin. A possible mechanism for regulating cadherin turnover. J Biol Chem. 2001;276(15):12301–9.

101. Lickert H, et al. Casein kinase II phosphorylation of E-cadherin increases E-cadherin/beta-catenin interaction and strengthens cell-cell adhesion. J Biol Chem. 2000;275(7):5090–5.
102. Wang R, et al. Activation of the Met receptor by cell attachment induces and sustains hepatocellular carcinomas in transgenic mice. J Cell Biol. 2001;153(5):1023–34.
103. Tward AD, et al. Distinct pathways of genomic progression to benign and malignant tumors of the liver. Proc Natl Acad Sci U S A. 2007;104(37):14771–6.
104. Tao J, et al. Modeling a human HCC subset in mice through co-expression of Met and point-mutant beta-catenin. Hepatology. 2016.
105. Howard S, et al. A positive role of cadherin in Wnt/beta-catenin signalling during epithelial-mesenchymal transition. PLoS One. 2011;6(8):e23899.
106. Polyak K, Weinberg RA. Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. Nat Rev Cancer. 2009;9(4):265–73.
107. Roussos ET, et al. AACR special conference on epithelial-mesenchymal transition and cancer progression and treatment. Cancer Res. 2010;70(19):7360–4.
108. Heuberger J, Birchmeier W. Interplay of cadherin-mediated cell adhesion and canonical Wnt signaling. Cold Spring Harb Perspect Biol. 2010;2(2):a002915.
109. Vermeulen L, et al. Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. Nat Cell Biol. 2010;12(5):468–76.
110. Korkaya H, Wicha MS. Cancer stem cells: nature versus nurture. Nat Cell Biol. 2010;12(5):419–21.
111. Brabletz T, et al. Variable beta-catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment. Proc Natl Acad Sci U S A. 2001;98(18):10356–61.
112. Huang FI, et al. Hepatocyte growth factor activates Wnt pathway by transcriptional activation of LEF1 to facilitate tumor invasion. Carcinogenesis. 2012;33(6):1142–8.
113. Fabregat I, Malfettone A, Soukupova J. New insights into the crossroads between EMT and stemness in the context of cancer. J Clin Med. 2016;5(3).
114. Yoshida K, et al. Hepatocyte growth factor-induced up-regulation of Twist drives epithelial-mesenchymal transition in a canine mammary tumour cell line. Res Vet Sci. 2014;97(3):521–6.
115. Gimbrone MA Jr, et al. Tumor dormancy in vivo by prevention of neovascularization. J Exp Med. 1972;136(2):261–76.
116. Brem S, et al. Prolonged tumor dormancy by prevention of neovascularization in the vitreous. Cancer Res. 1976;36(8):2807–12.
117. Chung AS, Lee J, Ferrara N. Targeting the tumour vasculature: insights from physiological angiogenesis. Nat Rev Cancer. 2010;10(7):505–14.
118. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell. 1996;86(3):353–64.
119. Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med. 1995;1(1):27–31.
120. Gerritsen ME. HGF and VEGF: a dynamic duo. Circ Res. 2005;96(3):272–3.
121. Sulpice E, et al. Cross-talk between the VEGF-A and HGF signalling pathways in endothelial cells. Biol Cell. 2009;101(9):525–39.
122. Xin X, et al. Hepatocyte growth factor enhances vascular endothelial growth factor-induced angiogenesis in vitro and in vivo. Am J Pathol. 2001;158(3):1111–20.
123. Van Belle E, et al. Potentiated angiogenic effect of scatter factor/hepatocyte growth factor via induction of vascular endothelial growth factor: the case for paracrine amplification of angiogenesis. Circulation. 1998;97(4):381–90.
124. Jaffe AB, Hall A. Rho GTPases: biochemistry and biology. Annu Rev Cell Dev Biol. 2005;21:247–69.
125. Connolly JO, et al. Rac regulates endothelial morphogenesis and capillary assembly. Mol Biol Cell. 2002;13(7):2474–85.
126. Royal I, et al. Activation of cdc42, rac, PAK, and rho-kinase in response to hepatocyte growth factor differentially regulates epithelial cell colony spreading and dissociation. Mol Biol Cell. 2000;11(5):1709–25.

127. Rosario M, Birchmeier W. How to make tubes: signaling by the Met receptor tyrosine kinase. Trends Cell Biol. 2003;13(6):328–35.
128. Gu H, Neel BG. The "Gab" in signal transduction. Trends Cell Biol. 2003;13(3):122–30.
129. Horiguchi N, et al. Hepatocyte growth factor promotes hepatocarcinogenesis through c-Met autocrine activation and enhanced angiogenesis in transgenic mice treated with diethylnitrosamine. Oncogene. 2002;21(12):1791–9.
130. Saucier C, et al. The Shc adaptor protein is critical for VEGF induction by Met/HGF and ErbB2 receptors and for early onset of tumor angiogenesis. Proc Natl Acad Sci U S A. 2004;101(8):2345–50.
131. Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. Nature. 2011;473(7347):298–307.
132. Jimenez B, et al. Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. Nat Med. 2000;6(1):41–8.
133. Zhang YW, et al. Hepatocyte growth factor/scatter factor mediates angiogenesis through positive VEGF and negative thrombospondin 1 regulation. Proc Natl Acad Sci U S A. 2003;100(22):12718–23.
134. Gao CF, et al. Proliferation and invasion: plasticity in tumor cells. Proc Natl Acad Sci U S A. 2005;102(30):10528–33.
135. Wang H, Keiser JA. Hepatocyte growth factor enhances MMP activity in human endothelial cells. Biochem Biophys Res Commun. 2000;272(3):900–5.
136. Koh SA, Lee KH. HGF mediated upregulation of lipocalin 2 regulates MMP9 through nuclear factor-kappaB activation. Oncol Rep. 2015;34(4):2179–87.
137. Monvoisin A, et al. Involvement of matrix metalloproteinase type-3 in hepatocyte growth factor-induced invasion of human hepatocellular carcinoma cells. Int J Cancer. 2002;97(2):157–62.
138. Giancotti FG, Ruoslahti E. Integrin signaling. Science. 1999;285(5430):1028–32.
139. Campbell ID, Humphries MJ. Integrin structure, activation, and interactions. Cold Spring Harb Perspect Biol. 2011;3(3).
140. Hynes RO. Integrins: bidirectional, allosteric signaling machines. Cell. 2002;110(6):673–87.
141. Avraamides CJ, Garmy-Susini B, Varner JA. Integrins in angiogenesis and lymphangiogenesis. Nat Rev Cancer. 2008;8(8):604–17.
142. Desgrosellier JS, Cheresh DA. Integrins in cancer: biological implications and therapeutic opportunities. Nat Rev Cancer. 2010;10(1):9–22.
143. Weis SM, Cheresh DA. alphaV integrins in angiogenesis and cancer. Cold Spring Harb Perspect Med. 2011;1(1):a006478.
144. Ephstein Y, et al. Critical role of S1PR1 and integrin beta4 in HGF/c-Met-mediated increases in vascular integrity. J Biol Chem. 2013;288(4):2191–200.
145. Ni X, et al. Interaction of integrin beta4 with S1P receptors in S1P- and HGF-induced endothelial barrier enhancement. J Cell Biochem. 2014;115(6):1187–95.
146. Hongu T, et al. Arf6 regulates tumour angiogenesis and growth through HGF-induced endothelial beta 1 integrin recycling. Nat Commun. 2015;6.
147. Hongu T, et al. Pathological functions of the small GTPase Arf6 in cancer progression: tumor angiogenesis and metastasis. Small GTPases. 2016;7(2):47–53.
148. Rahman S, et al. Novel hepatocyte growth factor (HGF) binding domains on fibronectin and vitronectin coordinate a distinct and amplified Met-integrin induced signalling pathway in endothelial cells. BMC Cell Biol. 2005;6(1):8.
149. Trusolino L, Bertotti A, Comoglio PM. A signaling adapter function for alpha6beta4 integrin in the control of HGF-dependent invasive growth. Cell. 2001;107(5):643–54.
150. Nikolopoulos SN, et al. Integrin beta4 signaling promotes tumor angiogenesis. Cancer Cell. 2004;6(5):471–83.
151. Zhao X, Guan JL. Focal adhesion kinase and its signaling pathways in cell migration and angiogenesis. Adv Drug Deliv Rev. 2011;63(8):610–5.

152. Chan PC, et al. Crosstalk between hepatocyte growth factor and integrin signaling pathways. J Biomed Sci. 2006;13(2):215–23.
153. Chen SY, Chen HC. Direct interaction of focal adhesion kinase (FAK) with Met is required for FAK to promote hepatocyte growth factor-induced cell invasion. Mol Cell Biol. 2006;26(13):5155–67.
154. Krock BL, Skuli N, Simon MC. Hypoxia-induced angiogenesis: good and evil. Genes Cancer. 2011;2(12):1117–33.
155. Bertout JA, Patel SA, Simon MC. The impact of O2 availability on human cancer. Nat Rev Cancer. 2008;8(12):967–75.
156. Hockel M, Vaupel P. Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. J Natl Cancer Inst. 2001;93(4):266–76.
157. Salceda S, Caro J. Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. J Biol Chem. 1997;272(36):22642–7.
158. Pugh CW, Ratcliffe PJ. Regulation of angiogenesis by hypoxia: role of the HIF system. Nat Med. 2003;9(6):677–84.
159. Liu Y, et al. Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Identification of a 5′ enhancer. Circ Res. 1995;77(3):638–43.
160. Tang N, et al. Loss of HIF-1alpha in endothelial cells disrupts a hypoxia-driven VEGF autocrine loop necessary for tumorigenesis. Cancer Cell. 2004;6(5):485–95.
161. Wang GL, Semenza GL. Characterization of hypoxia-inducible factor 1 and regulation of DNA binding activity by hypoxia. J Biol Chem. 1993;268(29):21513–8.
162. Eckerich C, et al. Hypoxia can induce c-Met expression in glioma cells and enhance SF/HGF-induced cell migration. Int J Cancer. 2007;121(2):276–83.
163. Kitajima Y, et al. Induction of hepatocyte growth factor activator gene expression under hypoxia activates the hepatocyte growth factor/c-Met system via hypoxia inducible factor-1 in pancreatic cancer. Cancer Sci. 2008;99(7):1341–7.
164. Yamamoto K, et al. Contribution of Bcl-2, but not Bcl-xL and Bax, to antiapoptotic actions of hepatocyte growth factor in hypoxia-conditioned human endothelial cells. Hypertension. 2001;37(5):1341–8.
165. Wang X, et al. Hepatocyte growth factor protects against hypoxia/reoxygenation-induced apoptosis in endothelial cells. J Biol Chem. 2003;279(7):5237–43.
166. Gilkes DM, Semenza GL, Wirtz D. Hypoxia and the extracellular matrix: drivers of tumour metastasis. Nat Rev Cancer. 2014;14(6):430–9.
167. Pugh CW, Ratcliffe PJ. The von Hippel-Lindau tumor suppressor, hypoxia-inducible factor-1 (HIF-1) degradation, and cancer pathogenesis. Semin Cancer Biol. 2003;13(1):83–9.
168. Gordon MS, et al. Safety, pharmacokinetics, and pharmacodynamics of AMG 102, a fully human hepatocyte growth factor-neutralizing monoclonal antibody, in a first-in-human study of patients with advanced solid tumors. Clin Cancer Res. 2010;16(2):699–710.
169. Rosen PJ, et al. A phase Ib study of AMG 102 in combination with bevacizumab or motesanib in patients with advanced solid tumors. Clin Cancer Res. 2010;16(9):2677–87.
170. Mok TS, et al. A randomized phase 2 study comparing the combination of Ficlatuzumab and Gefitinib with Gefitinib alone in Asian patients with advanced stage pulmonary adenocarcinoma. J Thorac Oncol. 2016;11(10):1736–44.
171. Patnaik A, et al. Phase I ficlatuzumab monotherapy or with erlotinib for refractory advanced solid tumours and multiple myeloma. Br J Cancer. 2014;111(2):272–80.
172. Houghton PJ, et al. Initial testing (Stage 1) of TAK-701, a humanized hepatocyte growth factor binding antibody, by the Pediatric Preclinical Testing Program. Pediatr Blood Cancer. 2014;61(2):380–2.
173. Okamoto W, et al. TAK-701, a humanized monoclonal antibody to hepatocyte growth factor, reverses gefitinib resistance induced by tumor-derived HGF in non-small cell lung cancer with an EGFR mutation. Mol Cancer Ther. 2010;9(10):2785–92.

174. Jin H, et al. MetMAb, the one-armed 5D5 anti-c-Met antibody, inhibits orthotopic pancreatic tumor growth and improves survival. Cancer Res. 2008;68(11):4360–8.
175. Tseng JR, et al. Preclinical efficacy of the c-Met inhibitor CE-355621 in a U87 MG mouse xenograft model evaluated by 18F-FDG small-animal PET. J Nucl Med. 2008;49(1):129–34.
176. Pacchiana G, et al. Monovalency unleashes the full therapeutic potential of the DN-30 anti-Met antibody. J Biol Chem. 2010;285(46):36149–57.
177. Liu L, et al. LY2875358, a neutralizing and internalizing anti-MET bivalent antibody, inhibits HGF-dependent and HGF-independent MET activation and tumor growth. Clin Cancer Res. 2014;20(23):6059–70.
178. Salgia R, et al. Phase I dose-escalation study of onartuzumab as a single agent and in combination with bevacizumab in patients with advanced solid malignancies. Clin Cancer Res. 2014;20(6):1666–75.
179. Diéras V, et al. Randomized, phase II, placebo-controlled trial of onartuzumab and/or bevacizumab in combination with weekly paclitaxel in patients with metastatic triple-negative breast cancer. Ann Oncol. 2015;26(9):1904–10.
180. Spigel DR, et al. Randomized phase II trial of Onartuzumab in combination with erlotinib in patients with advanced non-small-cell lung cancer. J Clin Oncol. 2013;31(32):4105–14.
181. Garber K. MET inhibitors start on road to recovery. Nat Rev Drug Discov. 2014;13(8):563–5.
182. Azuma K, et al. Phase II study of erlotinib plus tivantinib (ARQ 197) in patients with locally advanced or metastatic EGFR mutation-positive non-small-cell lung cancer just after progression on EGFR-TKI, gefitinib or erlotinib. ESMO Open. 2016;1(4):e000063.
183. Scagliotti G, et al. Phase III multinational, randomized, double-blind, placebo-controlled study of Tivantinib (ARQ 197) plus Erlotinib versus Erlotinib alone in previously treated patients with locally advanced or metastatic nonsquamous non-small-cell lung cancer. J Clin Oncol. 2015;33(24):2667–74.
184. Eng C, et al. A randomized, placebo-controlled, phase 1/2 study of tivantinib (ARQ 197) in combination with irinotecan and cetuximab in patients with metastatic colorectal cancer with wild-type KRAS who have received first-line systemic therapy. Int J Cancer. 2016;139(1):177–86.
185. Tolaney SM, et al. Phase II study of tivantinib (ARQ 197) in patients with metastatic triple-negative breast cancer. Invest New Drugs. 2015;33(5):1108–14.
186. Trojan J, Zeuzem S. Tivantinib in hepatocellular carcinoma. Expert Opin Investig Drugs. 2013;22(1):141–7.
187. Porta C, et al. Tivantinib (ARQ197) in hepatocellular carcinoma. Expert Rev Anticancer Ther. 2015;15(6):615–22.
188. Lee J, Tran P, Klempner SJ. Targeting the MET pathway in gastric and oesophageal cancers: refining the optimal approach. Clin Oncol (R Coll Radiol). 2016;28(8):e35–44.
189. Brandes F, et al. Targeting cMET with INC280 impairs tumour growth and improves efficacy of gemcitabine in a pancreatic cancer model. BMC Cancer. 2015;15:71.
190. Gavine PR, et al. Volitinib, a potent and highly selective c-Met inhibitor, effectively blocks c-Met signaling and growth in c-MET amplified gastric cancer patient-derived tumor xenograft models. Mol Oncol. 2015;9(1):323–33.
191. Rodig SJ, Shapiro GI. Crizotinib, a small-molecule dual inhibitor of the c-Met and ALK receptor tyrosine kinases. Curr Opin Investig Drugs. 2010;11(12):1477–90.
192. Kazandjian D, et al. FDA approval summary: crizotinib for the treatment of metastatic non-small cell lung cancer with anaplastic lymphoma kinase rearrangements. Oncologist. 2014;19(10):e5–11.
193. Schwab R, et al. Major partial response to crizotinib, a dual MET/ALK inhibitor, in a squamous cell lung (SCC) carcinoma patient with de novo c-MET amplification in the absence of ALK rearrangement. Lung Cancer. 2014;83(1):109–11.
194. Yakes FM, et al. Cabozantinib (XL184), a novel MET and VEGFR2 inhibitor, simultaneously suppresses metastasis, angiogenesis, and tumor growth. Mol Cancer Ther. 2011;10(12):2298–308.

195. Krajewska J, Olczyk T, Jarzab B. Cabozantinib for the treatment of progressive metastatic medullary thyroid cancer. Expert Rev Clin Pharmacol. 2016;9(1):69–79.
196. Singh H, et al. U.S. Food and Drug Administration approval: Cabozantinib for treatment of advanced renal cell carcinoma. Clin Cancer Res. 2016.
197. Neal JW, et al. Erlotinib, cabozantinib, or erlotinib plus cabozantinib as second-line or third-line treatment of patients with EGFR wild-type advanced non-small-cell lung cancer (ECOG-ACRIN 1512): a randomised, controlled, open-label, multicentre, phase 2 trial. Lancet Oncol. 2016;17(12):1661–71.
198. Drilon A, et al. Cabozantinib in patients with advanced RET-rearranged non-small-cell lung cancer: an open-label, single-centre, phase 2, single-arm trial. Lancet Oncol. 2016;17(12):1653–60.
199. Smith M, et al. Phase III Study of Cabozantinib in previously treated metastatic castration-resistant prostate cancer: COMET-1. J Clin Oncol. 2016;34(25):3005–13.
200. Tolaney SM, et al. Phase II and biomarker study of Cabozantinib in metastatic triple-negative breast cancer patients. Oncologist. 2016.
201. Qian F, et al. Inhibition of tumor cell growth, invasion, and metastasis by EXEL-2880 (XL880, GSK1363089), a novel inhibitor of HGF and VEGF receptor tyrosine kinases. Cancer Res. 2009;69(20):8009–16.
202. Kataoka Y, et al. Foretinib (GSK1363089), a multi-kinase inhibitor of MET and VEGFRs, inhibits growth of gastric cancer cell lines by blocking inter-receptor tyrosine kinase networks. Invest New Drugs. 2012;30(4):1352–60.
203. Chen HM, Tsai CH, Hung WC. Foretinib inhibits angiogenesis, lymphangiogenesis and tumor growth of pancreatic cancer in vivo by decreasing VEGFR-2/3 and TIE-2 signaling. Oncotarget. 2015;6(17):14940–52.
204. Choueiri TK, et al. Phase II and biomarker study of the dual MET/VEGFR2 inhibitor foretinib in patients with papillary renal cell carcinoma. J Clin Oncol. 2013;31(2):181–6.
205. Logan TF. Foretinib (XL880): c-MET inhibitor with activity in papillary renal cell cancer. Curr Oncol Rep. 2013;15(2):83–90.
206. Rayson D, et al. Canadian Cancer Trials Group IND197: a phase II study of foretinib in patients with estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2-negative recurrent or metastatic breast cancer. Breast Cancer Res Treat. 2016;157(1):109–16.
207. Seiwert T, et al. Phase II trial of single-agent foretinib (GSK1363089) in patients with recurrent or metastatic squamous cell carcinoma of the head and neck. Invest New Drugs. 2013;31(2):417–24.
208. Shah MA, et al. Phase II study evaluating 2 dosing schedules of oral foretinib (GSK1363089), cMET/VEGFR2 inhibitor, in patients with metastatic gastric cancer. PLoS One. 2013;8(3):e54014.
209. Shapiro GI, et al. A phase 1 dose-escalation study of the safety and pharmacokinetics of once-daily oral foretinib, a multi-kinase inhibitor, in patients with solid tumors. Invest New Drugs. 2013;31(3):742–50.
210. Yau TC, et al. A phase I/II multicenter study of single-agent Foretinib as first-line therapy in patients with advanced hepatocellular carcinoma. Clin Cancer Res. 2016.
211. Molife LR, et al. A phase I, dose-escalation study of the multitargeted receptor tyrosine kinase inhibitor, golvatinib, in patients with advanced solid tumors. Clin Cancer Res. 2014;20(24):6284–94.
212. Nakagawa T, et al. Lenvatinib in combination with golvatinib overcomes hepatocyte growth factor pathway-induced resistance to vascular endothelial growth factor receptor inhibitor. Cancer Sci. 2014;105(6):723–30.
213. Kosciuczuk EM, et al. Merestinib blocks Mnk kinase activity in acute myeloid leukemia progenitors and exhibits antileukemic effects in vitro and in vivo. Blood. 2016;128(3):410–4.
214. Bessudo A, et al. Phase I results of the randomized, placebo controlled, phase I/II study of the novel oral c-MET inhibitor, ARQ 197, irinotecan (CPT-11), and cetuximab (C) in patients

(pts) with wild-type (WT) KRAS metastatic colorectal cancer (mCRC) who have received front-line systemic therapy. J Clin Oncol. 2011;29(15_suppl):3582.
215. Yoshioka H, et al. A randomized, double-blind, placebo-controlled, phase III trial of erlotinib with or without a c-Met inhibitor tivantinib (ARQ 197) in Asian patients with previously treated stage IIIB/IV nonsquamous nonsmall-cell lung cancer harboring wild-type epidermal growth factor receptor (ATTENTION study). Ann Oncol. 2015;26(10):2066–72.
216. Koeppen H, et al. Biomarker analyses from a placebo-controlled phase II study evaluating erlotinib±onartuzumab in advanced non-small cell lung cancer: MET expression levels are predictive of patient benefit. Clin Cancer Res. 2014;20(17):4488–98.
217. Spigel DR, et al. Treatment Rationale Study Design for the MetLung Trial: a randomized, double-blind phase III study of Onartuzumab (MetMAb) in combination with Erlotinib versus Erlotinib alone in patients who have received standard chemotherapy for Stage IIIB or IV Met-positive non-small-cell lung cancer. Clin Lung Cancer. 2012;13(6):500–4.
218. Vashishtha A, et al. Safety data and patterns of progression in met diagnostic subgroups in OAM4558g; a phase II trial evaluating MetMAb in combination with erlotinib in advanced NSCLC. J Clin Oncol. 2011;29(15_suppl):7604.
219. Yu W, et al. Exploratory biomarker analyses from OAM4558g: a placebo-controlled phase II study of erlotinib with or without MetMAb in patients with advanced non-small cell lung cancer (NSCLC). J Clin Oncol. 2011;29(15_suppl):7529.
220. Puzanov I, et al. Phase 1 trial of tivantinib in combination with sorafenib in adult patients with advanced solid tumors. Invest New Drugs. 2015;33(1):159–68.
221. Ciamporcero E, et al. Combination strategy targeting VEGF and HGF/c-met in human renal cell carcinoma models. Mol Cancer Ther. 2015;14(1):101–10.
222. Subbiah V, et al. Activity of c-Met/ALK inhibitor Crizotinib and multi-kinase VEGF inhibitor Pazopanib in metastatic gastrointestinal neuroectodermal tumor harboring EWSR1-CREB1 fusion. Oncology. 2016;91(6):348–53.
223. Adjei AA, et al. Efficacy in selected tumor types in a phase I study of the c-MET inhibitor ARQ 197 in combination with sorafenib. J Clin Oncol. 2011;29(15_suppl):3034.
224. Eder JP, et al. Novel therapeutic inhibitors of the c-Met signaling pathway in cancer. Clin Cancer Res. 2009;15(7):2207–14.

Chapter 9
Pericellular Activation of Peptide Growth Factors by Serine Proteases

Hiroaki Kataoka and Tsuyoshi Fukushima

Abstract The growth, survival, and metabolic activities of multicellular organisms at the cellular level are regulated not only by intracellular signal transduction pathways but also by systemic homeostasis and the pericellular microenvironment. The significance of the pericellular microenvironment is also established in tumorigenesis and malignant progression of transformed cells, in which processing of bioactive molecules by extracellular proteases has significant roles. Proteolytic activation of peptide growth factors in the pericellular microenvironment enables the induction of outside-in signaling in constituent cells in both physiological and pathological settings. This chapter will review the current knowledge of pericellular activation of peptide growth factors by serine proteases, with the main focus on activation of hepatocyte growth factor (HGF) that transduces signals through the MET receptor tyrosine kinase. There are two mechanisms for HGF activation *in vivo*: serum activation and cellular activation. Type II transmembrane serine proteases are membrane-anchored proteases that are part of cellular HGF-activating machinery. In the past decade, evidence for the roles of these proteases in cancer progression has been rapidly emerging.

Keywords Protease • HGF • TTSP • Matriptase • Hepsin • HAI-1 • HAI-2

9.1 Growth Factors That Require Extracellular Proteolytic Activation

Many intracellular pathways execute posttranscriptional modification of synthesized proteins, including glycosylation, phosphorylation, ubiquitination, SUMOylation, and sulfurylation. However, in extracellular spaces, tools for the

H. Kataoka (✉) • T. Fukushima
Section of Oncopathology and Regenerative Biology, Department of Pathology, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan
e-mail: mejina@med.miyazaki-u.ac.jp

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N. Shinomiya et al. (eds.), *Regulation of Signal Transduction in Human Cell Research*, Current Human Cell Research and Applications,
https://doi.org/10.1007/978-981-10-7296-3_9

modulation of secreted proteins are limited, and thus protein degradation occurring through proteolytic processing is a major modality to alter protein structure and function. Peptide growth factors are proteins that transduce signals into cells that express their specific receptors, and this process is critically involved in many developmental, physiological, and pathological phenomena *in vivo*. These growth factors are secreted from cells or expressed on cell surfaces; however, in many cases, they must be processed by proteases to establish an efficient paracrine or autocrine stimulation loop (Table 9.1). For example, transforming growth factor (TGF)-β complexes with latency-associated peptide (LAP) in the extracellular milieu. Whereas integrin-mediated conformational change of the complex is critical for the activation, protease-mediated LAP degradation accelerates the activation process [1] (Fig. 9.1a). Meanwhile, some epidermal growth factor (EGF) receptor ligands such as heparin-binding EGF-like growth factor (HB-EGF), TGF-α, and amphiregulin are expressed as membrane-bound growth factors, and proteolytic shedding is required to transduce their signals to cells apart from the producing cells. Matrix metalloproteases (MMPs), the disintegrin and metalloprotease (ADAM) family (particularly ADAM-17), and the serine protease plasmin have all been proposed to participate in the degradation of inhibitory proteins or the shedding of membrane-bound growth factors [2–4] (Table 9.1). Among MMPs, membrane-bound type I MMP (MMP-14) is preferentially expressed in neoplastic tissues and has a significant role in activating peptide growth factors in cancer tissues [5]. On the other hand, some peptide growth factors are secreted as inactive precursor forms, such that activation by limited proteolysis is critically required for their function (Fig. 9.1b). This group of growth factors includes hepatocyte growth factor

Table 9.1 Examples of growth factor activation in cancer microenvironment

Growth factors	Proteases	Mechanism of activation
IGF-1, -2	MMP-7, -3; ADAM-28	Degradation of complexed protein (IGF binding protein)
VEGF-A	MMP-1, -3, -13; ADAM-28	Degradation of complexed protein (connective tissue growth factor)
TGF-β	MT1-MMP; MMP-13; <u>Plasmin</u>	Degradation of complexed protein (latency-associated peptide)
HB-EGF	ADAM-17 and other ADAMs; MMP-7; MT1-MMP	Shedding of extracellular domain
Amphiregulin TGF-α	ADAM-17	Shedding of extracellular domain
HGF/SF	<u>HGFAc</u>; <u>Matriptase</u>; <u>Hepsin</u>; <u>TMPRSS13</u>; <u>TMPRSS11D</u>	Conversion of proform to active form by limited proteolysis
MSP	<u>HGFAc</u>; <u>Matriptase</u>; <u>TMPRSS11D</u>	Conversion of proform to active form by limited proteolysis
PDGF-C	<u>Matriptase</u>; <u>uPA</u>	Conversion of proform to active form by limited proteolysis
PDGF-D	<u>Matriptase</u>	Conversion of proform to active form by limited proteolysis

Serine proteases are underlined

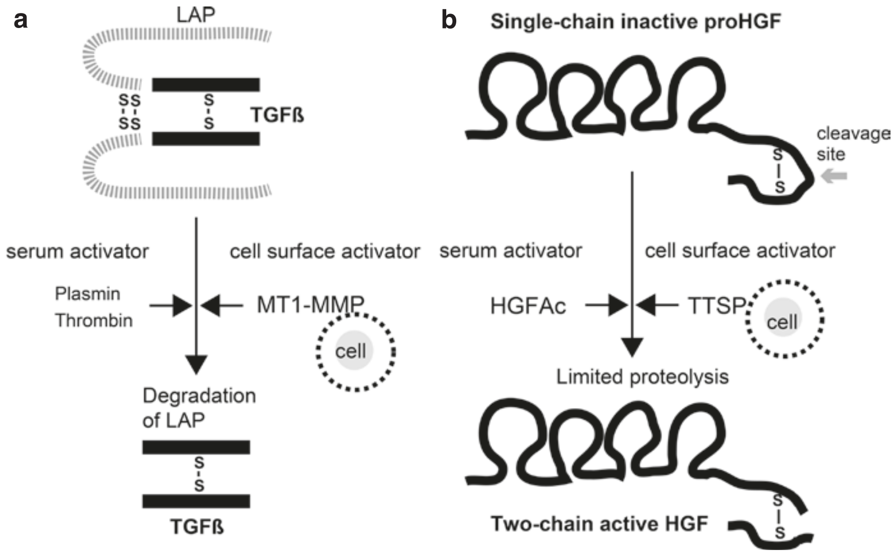

Fig. 9.1 Examples of extracellular activation of peptide growth factors. (**a**) Activation of TGF-β by proteolytic degradation of the inhibitory binding protein, LAP. (**b**) Activation of HGF by limited proteolysis of inactive pro-HGF. In both cases, serum protease (i.e., serum activator) and cell surface protease (i.e., cell surface activator) can be involved in activation

(HGF), macrophage stimulating protein (MSP), platelet-derived growth factor (PDGF)-C, and PDGF-D (Table 9.1). These peptide growth factors have a basic amino acid at the P1 position in the activation cleavage site, suggesting that a trypsin-like extracellular protease is responsible for the induced activation.

In 1992, Shimomura et al. identified a very efficient HGF-activating activity in fetal bovine serum (FBS)-supplemented culture-conditioned medium of Chinese hamster ovary cells and purified a novel serine protease from FBS [6]. This activity resulted in the cleavage of the Arg494-Val495 bond in the HGF precursor (pro-HGF) to generate a two-chain active form of HGF that transduces signals upon binding to its specific receptor tyrosine kinase MET [7]. This serum pro-HGF-activating protease was designated as HGF activator (HGFAc), and its human counterpart was purified from human serum and the cDNA was cloned [8]. HGFAc is homologous to coagulation factor XII and is synthesized mainly by the liver and circulates as a zymogen (pro-HGFAc) [9]. At sites of tissue injury, thrombin activates circulating pro-HGFAc [10] and induces a robust activation of pro-HGF, which is required for the subsequent regeneration phase [9, 11]. Serum HGFAc is also an efficient activator of MSP that has structural homology with HGF and serves as a ligand of the Receptor d'Origine Nantais (RON) receptor tyrosine kinase. Upon activation by HGFAc, MSP might be involved in regulating macrophage functions at the sites of tissue injury and inflammation [12, 13].

On the other hand, to maintain homeostasis and physiological activity, a localized, serum-independent activation system of these growth factors is also necessary

for a normal tissue microenvironment and also in the developmental processes. Moreover, even in pathological conditions, localized pericellular growth factor activation is needed; for example, invading cancer cells require outside-in signals mediated by autocrine and paracrine growth factors such as HGF for motility and survival [14, 15]. HGF-induced paracrine signaling via MET is also necessary to maintain cancer stem cells [16, 17]. Thus, there is rapidly accumulating evidence to indicate that membrane-anchored serine proteases are key components of cell machinery involved in the activation of growth factors, including HGF, in the pericellular microenvironment [18, 19].

9.2 Membrane-Anchored Serine Proteases

Type II transmembrane serine proteases (TTSPs) form a large family of membrane-anchored proteases, and in humans the family includes 17 serine proteases [18, 20] (Fig. 9.2). TTSPs have a single-pass transmembrane domain near the N-terminus and a short intracellular domain. The longer extracellular portion contains a conserved serine protease domain that has the catalytic triad of His, Asp, and Ser characteristic of the S1 fold subfamily of peptidases. All TTSPs have a cleavage preference for substrates with Arg or Lys (basic amino acid) in the P1 position [18].

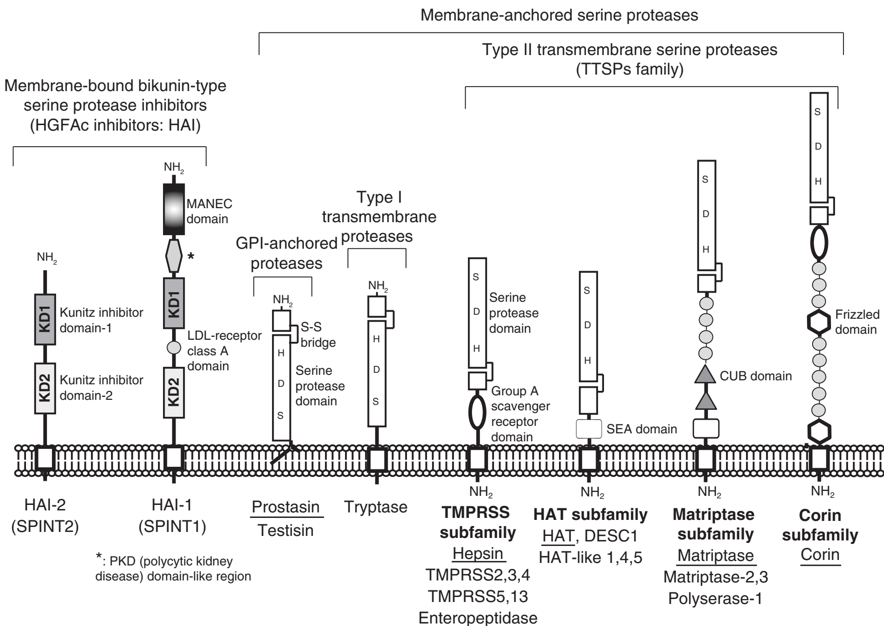

Fig. 9.2 Domain structures of human membrane-anchored serine proteases and membrane-anchored serine protease inhibitors (HAI-1, HAI-2)

They can be divided into some subfamilies depending on additional domain structures, for example, matriptase subfamily four low-density lipoprotein (LDL)-receptor class A domains; two complement C1r/C1s, Uegf, Bmp1 (CUB) domains; and a sperm protein, enterokinase, and agrin (SEA) domain (Fig. 9.2). The cell surface localization of TTSPs is reasonably advantageous for processing pericellular or cell surface bioactive molecules. In fact, the processing functions of some TTSPs are critical for maintaining normal homeostasis. For example, corin is essentially required for the activation of atrial natriuretic peptide that has roles in cardiovascular and renal functions [21]. Matriptase is expressed in most epithelial cells and is essential for epithelial integrity and barrier function [22]. Hemojuvelin processing by transmembrane protease, serine 6 (TMPRSS6, also known as matriptase-2), is a key step in iron metabolism, and defects in TMPRSS6/matriptase-2 activity are associated with iron-refractory iron deficiency anemia [23]. Furthermore, researchers are drawing attention to the roles of TTSPs in pathological conditions such as cancer [20]. In addition to TTSPs, type I transmembrane protease and glycosylphosphatidylinositol (GPI)-anchored proteases are known as membrane-anchored serine proteases (Fig. 9.2), and interactions between these proteases and TTSPs are present. The interaction between matriptase and prostasin is the well-known example of this interplay; matriptase activates prostasin and vice versa [18].

9.3 Emerging Roles of TTSPs in Cancer

The cancer cell microenvironment plays a critical role in malignant progression and consists of a complex mixture of neoplastic cells and host-derived stromal cells, such as immune/inflammatory cells, cancer-associated fibroblasts (CAF), and endothelial cells [14]. In this microenvironment, extracellular matrix proteins as well as matricellular and cellular proteins in cancer cells and stromal cells are modified, and pericellular proteolysis plays a significant role in the degradation, activation, or other processing to reveal latent activity of these proteins. These reactions in cancer tissues resemble those involved in inflammation and wound repair such that tumors are sometimes referred to as “wounds that never heal” [24]. A mutual interaction between cancer cells and stroma cells can orchestrate enhanced growth factor production and activity in tumor tissue. For example, cancer cells stimulate CAFs to produce pro-HGF that is subsequently activated by pericellular HGF-activating protease to induce MET-induced signaling in cancer cells and endothelial cells [7, 15]. Therefore, extracellular activation of paracrine peptide growth factors by proteases is a key step in facilitating mutual interactions between cancer cells and stromal cells.

Recently, increasing evidence has highlighted how carcinogenesis and malignant progression of tumors is aided by TTSPs, with particular focus on matriptase, hepsin, TMPRSS2, and TMPRSS4 [18, 20, 25]. The proposed mechanisms by which these TTSPs facilitate cancer progression involve activation of bioactive molecules such as peptide growth factors and other zymogens, as well as protease-activated

receptor 2 (PAR-2) [18, 20]. Degradation of extracellular matrix protein such as laminin is also proposed. However, other as yet unknown mechanisms may also have a role [25, 26]. Nonetheless, pericellular activation of pro-HGF by TTSPs, particularly matriptase and hepsin, has been of significant interest to researchers studying cancer-related TTSPs.

9.4 Pericellular Activation of HGF by TTSPs in Tumor Tissues

As already discussed, there are two major mechanisms for pro-HGF processing *in vivo*: (a) triggering of the serum activation system (e.g., HGFAc) after tissue injury accompanied by thrombin generation and (b) triggering of the cellular activation system (e.g., TTSPs). Although both systems likely function in activating HGF in cancer tissues [7, 19, 27, 28], the cellular activation system could have superior significance for carcinogenesis and also for cancer cells in hypovascular regions or the invasion front with a fibrotic stromal reaction. Indeed, localized pericellular activation of HGF is reported to be important for cancer cells to evade apoptosis, maintain a stem cell-like phenotype, and invade the stroma [15]. Among TTSPs, matriptase, hepsin, TMPRSS13, and the human airway trypsin-like protease (HAT) are known to have pro-HGF processing activity [18, 29, 30]. Because of the molecular similarity between HGF and MSP, all of these HGF activators also activate MSP to transduce RON-mediated signaling to macrophages, epithelial cells, and cancer cells. In fact, HGFAc and matriptase are major serum and cellular activators of MSP, respectively [12, 31], and hepsin and HAT also serve as cellular activators [32, 33].

Matriptase, which was initially identified in breast cancer cells [34] and is also known as membrane-type serine protease 1 (MT-SP1) [35], is one of the most studied TTSPs [18, 20]. Among HGF-activating TTSPs, matriptase likely exerts the highest specific activity for pro-HGF processing [36], and this activity might be highly important in cancer biology. In mice, transgenic matriptase expression in epidermal keratinocytes under control of the keratin 5 promoter causes squamous cell carcinoma [37]. This phenomenon is mediated by the MET-AKT-mTOR (mammalian target of rapamycin) signaling axis initiated by matriptase-mediated HGF activation [28]. Consequently, head and neck squamous cell carcinoma patients with high matriptase expression tend to have a worse prognosis [38]. Inflammatory breast cancer, a subtype of breast cancer with poor prognosis, likely uses the matriptase/HGF/MET axis for invasive growth [39]. Another example of matriptase involvement in cancers is high-grade malignant B-cell lymphoma wherein diffuse large B-cell lymphoma and Burkitt lymphoma cells frequently overexpress matriptase and show enhanced pericellular HGF activation [40]. Consequently, inhibition of matriptase activity significantly suppresses the proliferation of lymphoma cells [40].

Hepsin is another efficient HGF-activating TTSP that is believed to be involved in cancer progression. Hepsin (also known as TMPRSS1) was initially identified in

liver and hepatocellular carcinoma (HCC) cells [41]. Earlier findings showed that suppression of hepsin expression inhibited the growth of several cultured cell lines *in vitro*. Therefore, hepsin was reported to be a protein required for mammalian cell growth [42]. However, the phenotype of hepsin knockout (KO) mice indicated that hepsin is not essential for embryonic development, normal morphology of major organs, or liver regeneration [43], although a subsequent study revealed that hepsin KO mice exhibit a profound hearing loss [44]. Regarding the role of hepsin in cancers, extensive studies have been done in prostatic cancers, in which hepsin enhances invasion and metastasis of prostatic cancer cells [45, 46]. Similar observations were reported for ovarian cancer cells [47]. Meanwhile, renal cell carcinoma patients with higher hepsin mRNA levels had a poorer prognosis [48]. The mechanisms by which hepsin promotes invasion and metastasis are as yet unclear. Whereas hepsin-mediated degradation of the extracellular matrix may be responsible for its proinvasive function [45], hepsin processing activities for pro-HGF and pro-MSP followed by signaling through MET and RON receptor tyrosine kinases may also underpin the enhanced invasion and metastasis mediated by hepsin [32, 47].

9.5 Other Molecules Regulated by TTSP-Mediated Activation in Tumor Tissues

Recent studies revealed that PDGF-C and PDGF-D are also matriptase-dependent peptide growth factors in cancer tissues and contribute to breast and prostatic cancer progression [49–51]. In prostatic cancer, PDGF-D levels were associated with both Gleason score and tumor stage. Moreover, PDGF-D induced activation and shedding of matriptase through β-PDGF receptor signaling [52]. Therefore, mutual amplifying interactions between PDGF-D signaling and matriptase-mediated reactions are likely established in the prostatic cancer microenvironment and may contribute to malignant progression. Moreover, matriptase efficiently activates PAR-2, a seven transmembrane α-helix G protein-coupled receptor that is expressed in cancer cells, vascular cells, and CAF [53–55], as well as other proteases that are known to be involved in cancer progression, such as urokinase-type plasminogen activator (uPA) [56]. Like matriptase, hepsin activates not only HGF but also MSP, uPA, and, notably, matriptase zymogen [53, 57].

9.6 Hepatocyte Growth Factor Activator Inhibitor (HAI): Regulators of TTSPs

HAI was initially purified from culture-conditioned medium of MKN45 gastric adenocarcinoma cells and was identified as a cell surface inhibitor of HGFAc [58, 59]. A second HAI was also purified from MKN45-conditioned medium [60]. These

protease inhibitors were designated as HAI-1 and HAI-2, and the current official symbols for these two inhibitors are SPINT1 (serine peptidase inhibitor, Kunitz type I) and SPINT2, respectively. cDNA cloning revealed that HAI-2/SPINT2 was identical to placental bikunin, which was reported independently by another group as a novel Kunitz-type inhibitor that is abundantly expressed in the placenta [61]. Both HAI-1/SPINT1 and HAI-2/SPINT2 are type I transmembrane proteins with two extracellular Kunitz-type serine protease inhibitor domains (Fig. 9.2). Therefore, their biological activities may be limited to the cellular surface or pericellular microenvironment. Although HAI-1/SPINT1 and HAI-2/SPINT2 show similar structural features, the N-terminus of HAI-1/SPINT1 has an N-terminus with eight cysteines (MANEC) domain as well as an LDL-receptor class A domain between the two Kunitz domains, both of which are absent in HAI-2/SPINT2 (Fig. 9.2). Despite the initial discovery of HAI-1/SPINT1 and HAI-2/SPINT2 as cellular inhibitors of HGFAc, recent studies demonstrated that both HAIs are critical regulators of TTSPs [18, 20].

HAI-1/SPINT1 is expressed in most epithelial cells and placental trophoblasts [62–64] and inhibits all the main HGF-activating serine proteases, including HGFAc, matriptase, hepsin, TMPRSS13, and HAT [18, 19]. Kallikrein 1-related peptidase (KLK)-4 and KLK-5 are also sensitive to HAI-1/SPINT1, and the ability of both KLKs to activate pro-HGFAc suggests an involvement in HGF-activating machinery *in vivo* [65, 66]. Among these proteases, matriptase is one of the most important cognate proteases of HAI-1/SPINT1 [18, 67]. Indeed, a matriptase-HAI-1/SPINT1 complex can be observed both in *in vitro* cell-based assays and in *in vivo* body fluids [67, 68]. Dysregulation of matriptase activity in the pericellular microenvironment occurs if cellular levels of HAI-1/SPINT1 are insufficient. In fact, reduced cell surface HAI-1/SPINT1 expression resulted in increased extracellular matriptase activity [69, 70], which enhanced the invasiveness of various types of carcinoma cells [26, 71–73] as well as infiltration of CAFs [55]. In accordance with these findings, matriptase-induced skin carcinogenesis in mice was significantly suppressed when Hai-1/Spint1 was concomitantly expressed in keratinocytes with induced matriptase expression [37]. In patients with oral squamous cell carcinoma (OSCC), decreased cell surface HAI-1/SPINT1 in the cancer cells correlated with a more invasive histology of the invasion front, increased number of CAFs, and the presence of lymph node metastasis [71]. Moreover, mice with intestine-specific deletion of *Spint1* showed increased tumorigenesis in a chemical carcinogen-induced colon cancer model and an *Apc* mutation-induced intestinal tumor model in mice, accompanying enhanced HGF activation in the intestinal mucosa [74, 75]. The insufficient HAI-1/SPINT1 function may arise in cancer cells not only by reduced *SPINT1* gene transcription but also by enhanced shedding by metalloproteases, including MT1-MMP, from the cell surface [26, 74, 76].

Like HAI-1, HAI-2 is also preferentially expressed in epithelial cells and the placenta and efficiently inhibits the major HGF-activating proteases [7, 20]. However, its role *in vivo* remains unclear. Recently, a missense mutation in the *SPINT2* gene was found to be associated with a syndromic form of congenital sodium diarrhea, indicating that HAI-2/SPINT2 has a crucial role in the intestinal

tract [77]. Indeed, HAI-2/SPINT2, but not HAI-1/SPINT1, is required for the proper cell surface localization and activation of matriptase acting as a specific molecular chaperone for matriptase trafficking in intestinal epithelial cells [78]. Therefore, the roles for HAIs in the regulation of TTSP functions are very complex depending on the cell type and situation, as is evidenced by the diverse expression patterns of HAI-2/SPINT2 in cancers. HAI-2/SPINT2 is significantly downregulated in HCC [79], renal cell carcinomas [80, 81], glioblastomas [82], medulloblastomas [83], and melanomas [84] by hypermethylation of the SPINT2 promoter. In these cancer cells, engineered restoration of HAI-2/SPINT2 expression suppressed the invasive growth of these cells, indicating that HAI-2/SPINT2 is a tumor suppressor. On the other hand, HAI-2 is overexpressed in some cancer types, such as pancreatic ductal adenocarcinoma [85]. In prostatic cancers, *SPINT2* mRNA levels were not decreased, but HAI-2/SPINT2 immunoreactivity was reduced [86], and HAI-2/SPINT2 suppressed the invasive growth of prostatic cancer cells by regulating matriptase activity [87]. Thus, there is clearly a need for further studies on the pathophysiological functions of HAI-2/SPINT2 to obtain a better picture of the dynamics that occur in cancer cells and the cancer tissue microenvironment.

9.7 Conclusion and Future Perspectives

The extracellular activation of growth factors and transduction of their signals through specific cell surface receptors must be a tightly regulated phenomenon that includes both positive and negative controls. This chapter introduces the crucial and complex roles of pericellular proteases in the activation of peptide growth factors, with a focus on HGF and molecules involved in HGF-activating machinery in tumors, in which matriptase, hepsin, HGFAc, and their inhibitors HAI-1/SPINT1 and HAI-2/SPINT2 have crucial roles (Fig. 9.3). Matriptase is also important in the pericellular activation of other growth factors such as MSP, PDGF-C, and PDGF-D. The pericellular activation system described in this chapter may thus be a promising tumor microenvironment target that can be exploited to control the malignant progression of the cancer cells. Furthermore, the N-terminal Kunitz domain (KD1) of HAI-1/SPINT1 is known to be the functional domain for inhibition of HGFAc and major HGF-activating TTSPs, and thus KD1 may be useful for targeting of these proteases. In fact, recombinant KD1 suppressed metastatic spreading of prostatic and pancreatic cancer cells in mouse models [46, 72]. Generation of neutralizing antibodies against these proteases may thus be a useful approach, and a specific antibody acting as an allosteric inhibitor of catalytic activity that binds at the periphery of the substrate binding site of the protease has been reported [88, 89]. This allosteric inhibition mechanism may have implications for the development of innovative strategies to regulate cancer-associated proteases such as TTSPs and HGFAc. Development of synthetic chemicals that specifically inhibit these HGF-activating proteases may provide a more sophisticated and cost-effective approach

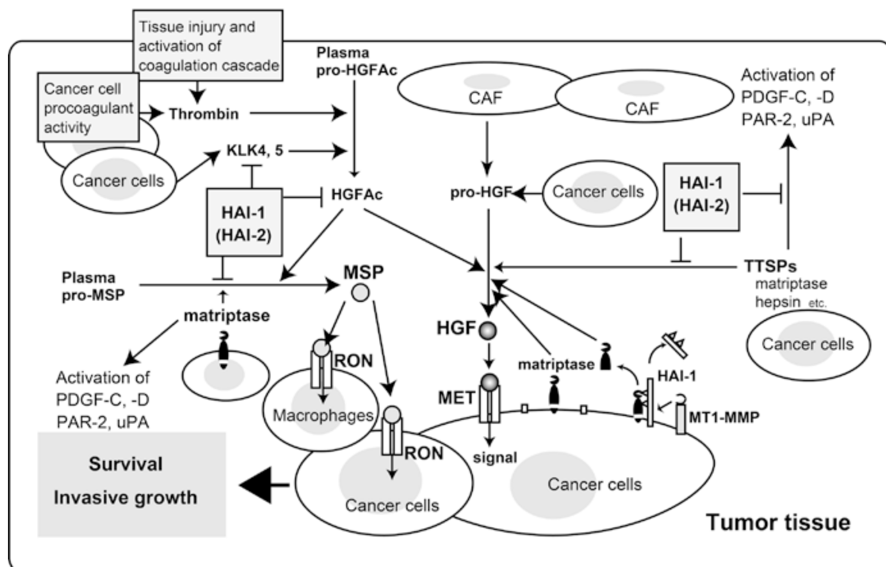

Fig. 9.3 Schematic drawing of pericellular activation of HGF and MSP in tumor tissues. Tissue injury-induced activation system mediated by HGFAc and cellular activation system mediated by TTSP such as matriptase and hepsin are functioning in the activation. Matriptase also activates PDGF-C, PDGF-D, uPA, and PAR-2, which may also contribute to malignant progression of the tumor

to control excess growth factor activities in cancer tissues. These agents are currently being investigated, and several promising compounds have already been reported [90–93].

Conflict of Interest The authors declare no financial and commercial conflicts of interest.

References

1. Shields MA, Dangi-Garimella S, Redig AJ, Munshi HG. Biochemical role of the collagen-rich tumour microenvironment in pancreatic cancer progression. Biochem J. 2012;441:541–52.
2. Koshikawa N, Mizushima H, Minegishi T, Eguchi F, Yotsumoto F, Nabeshima K, et al. Proteolytic activation of heparin-binding EGF-like growth factor by membrane-type matrix metalloproteinase-1 in ovarian carcinoma cells. Cancer Sci. 2011;102:111–6.
3. Taylor SR, Markesbery MG, Harding PA. Heparin-binding epidermal growth factor-like growth factor (HB-EGF) and proteolytic processing by a disintegrin and metalloproteinases (ADAM): a regulator of several pathways. Semin Cell Dev Biol. 2014;28:22–30.
4. Cataisson C, Michalowski AM, Shibuya K, Ryscavage A, Klosterman M, Wright L, et al. MET signaling in keratinocytes activates EGFR and initiates squamous carcinogenesis. Sci Signal. 2016;9(433):ra62.
5. Domoto T, Takino T, Guo L, Sato H. Cleavage of hepatocyte growth factor activator inhibitor-1 by membrane-type MMP-1 activates matriptase. Cancer Sci. 2012;103:448–54.

6. Shimomura T, Ochiai M, Kondo J, Morimoto Y. A novel protease obtained from FBS-containing culture supernatant, that processes single chain form hepatocyte growth factor to two chain form in serum-free culture. Cytotechnology. 1992;8:219–29.
7. Kataoka H, Miyata S, Uchinokura S, Itoh H. Roles of hepatocyte growth factor (HGF) activator and HGF activator inhibitor in the pericellular activation of HGF/scatter factor. Cancer Metastasis Rev. 2003;22:223–36.
8. Miyazawa K, Shimomura T, Kitamura A, Kondo J, Morimoto Y, Kitamura N. Molecular cloning and sequence analysis of the cDNA for a human serine protease responsible for activation of hepatocyte growth factor. Structural similarity of the protease precursor to blood coagulation factor XII. J Biol Chem. 1993;268:10024–8.
9. Kataoka H, Kawaguchi M. Hepatocyte growth factor activator (HGFA): pathophysiological functions in vivo. FEBS J. 2010;277:2230–7.
10. Shimomura T, Kondo J, Ochiai M, Naka D, Miyazawa K, Morimoto Y, et al. Activation of the zymogen of hepatocyte growth factor activator by thrombin. J Biol Chem. 1993;268:22927–32.
11. Itoh H, Naganuma S, Takeda N, Miyata S, Uchinokura S, Fukushima T, et al. Regeneration of injured intestinal mucosa is impaired in hepatocyte growth factor activator-deficient mice. Gastroenterology. 2004;127:1423–35.
12. Kawaguchi M, Orikawa H, Baba T, Fukushima T, Kataoka H. Hepatocyte growth factor activator is a serum activator of single-chain precursor macrophage-stimulating protein. FEBS J. 2009;276:3481–90.
13. Li J, Chanda D, Shiri-Sverdlov R, Neumann D. MSP: an emerging player in metabolic syndrome. Cytokine Growth Factor Rev. 2015;26:75–82.
14. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. Cancer Cell. 2012;21:309–22.
15. Gherardi E, Birchmeier W, Birchmeier C, Vande Woude G. Targeting MET in cancer: rationale and progress. Nat Rev Cancer. 2012;12:89–103.
16. Vermeulen L, De Sousa E, Melo F, van der Heijden M, Cameron K, de Jong JH, Borovski T, et al. Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. Nat Cell Biol. 2010;12:468–76.
17. Lau EYT, Lo J, Cheng BYL, Ma MKF, Lee JMF, Ng JKY, et al. Cancer-associated fibroblasts regulate tumor-initiating cell plasticity in hepatocellular carcinoma through c-Met/FRA1/HEY1 signaling. Cell Rep. 2016;15:1175–89.
18. Antalis TM, Buzza MS, Hodge KM, Hooper JD, Netzel-Arnett S. The cutting edge: membrane-anchored serine protease activities in the pericellular microenvironment. Biochem J. 2010;428:325–46.
19. Kawaguchi M, Kataoka H. Mechanisms of hepatocyte growth factor activation in cancer tissues. Cancers (Basel). 2014;6:1890–04.
20. Tanabe LM, List K. The role of type II transmembrane serine protease mediated signaling in cancer. FEBS J. 2016. https://doi.org/10.1111/febs.13971.
21. Yan W, Wu F, Morser J, Wu Q. Corin, a transmembrane cardiac serine protease, acts as a pro-atrial natriuretic peptide-converting enzyme. Proc Natl Acad Sci U S A. 2000;97:8525–9.
22. Miller GS, List K. The matriptase-prostasin proteolytic cascade in epithelial development and pathology. Cell Tissue Res. 2013;351:245–53.
23. Wang CY, Meynard D, Lin HY. The role of TMPRSS6/matriptase-2 in iron regulation and anemia. Front Pharmacol. 2014;5:114.
24. Dvorak HF. Tumors: wounds that do not heal. similarities between tumor stroma generation and wound healing. N Engl J Med. 1986;315:1650–9.
25. De Aberasturi AL, Calvo A. TMPRSS4: an emerging potential therapeutic target in cancer. Br J Cancer. 2015;112:4–8.
26. Cheng H, Fukushima T, Takahashi N, Tanaka H, Kataoka H. Hepatocyte growth factor activator inhibitor type 1 regulates epithelial to mesenchymal transition through membrane-bound serine proteinases. Cancer Res. 2009;69:1828–35.

27. Kataoka H, Hamasuna R, Itoh H, Kitamura N, Koono M. Activation of hepatocyte growth factor/scatter factor in colorectal carcinoma. Cancer Res. 2000;60:6148–59.
28. Szabo R, Rasmussen AL, Moyer AB, Kosa P, Schafer JM, Molinolo AA, et al. c-Met-induced epithelial carcinogenesis is initiated by the serine protease matriptase. Oncogene. 2011;30:2003–16.
29. Hashimoto T, Kato M, Shimomura T, Kitamura N. TMPRSS13, a type II transmembrane serine protease, is inhibited by hepatocyte growth factor activator inhibitor type 1 and activates pro-hepatocyte growth factor. FEBS J. 2010;277:4888–900.
30. Kato M, Hashimoto T, Shimomura T, Kataoka H, Ohi H, Kitamura N. Hepatocyte growth factor activator inhibitor type 1 inhibits protease activity and proteolytic activation of human airway trypsin-like protease. J Biochem. 2012;151:179–87.
31. Bhatt AS, Welm A, Farady CJ, Vasquez M, Wilson K, Craik CS. Coordinate expression and functional profiling identify an extracellular proteolytic signaling pathway. Proc Natl Acad Sci U S A. 2007;104:5771–6.
32. Ganesan R, Kolumam GA, Lin SJ, Xie M-H, Santell L, TD W, et al. Proteolytic activation of pro-macrophage-stimulating protein by hepsin. Mol Cancer Res. 2011;9:1175–86.
33. Orikawa H, Kawaguchi M, Baba T, Yorita K, Sakoda S, Kataoka H. Activation of macrophage-stimulating protein by human airway trypsin-like protease. FEBS Lett. 2012;586:217–21.
34. Lin CY, Wang JK, Torri J, Dou L, Sang QA, Dickson RB. Characterization of a novel, membrane-bound, 80-kDa matrix-degrading protease from human breast cancer cells. Monoclonal antibody production, isolation, and localization. J Biol Chem. 1997;272:9147–52.
35. Takeuchi T, Shuman MA, Craik CS. Reverse biochemistry: use of macromolecular protease inhibitors to dissect complex biological processes and identify a membrane-type serine protease in epithelial cancer and normal tissue. Proc Natl Acad Sci U S A. 1999;96:11054–61.
36. Owen KA, Qiu D, Alves J, Schumacher AM, Kilpatrick LM, Li J, et al. Pericellular activation of hepatocyte growth factor by the transmembrane serine proteases matriptase and hepsin, but not by the membrane-associated protease uPA. Biochem J. 2010;426:219–28.
37. List K, Szabo R, Molinolo A, Sriuranpong V, Redeye V, Murdock T, et al. Deregulated matriptase causes ras-independent multistage carcinogenesis and promotes ras-mediated malignant transformation. Genes Dev. 2005;19:1934–50.
38. Cheng MF, Huang MS, Lin CS, Lin LH, Lee HS, Jiang JC, Hsia KT. Expression of matriptase correlates with tumour progression and clinical prognosis in oral squamous cell carcinoma. Histopathology. 2014;65:24–34.
39. Zoratti GL, Tanabe LM, Hyland TE, Duhaime MJ, Colombo É, Leduc R, Marsault E, Johnson MD, Lin C-Y, Boerner J, Lang JE, List K. Matriptase regulates c-Met mediated proliferation and invasion in inflammatory breast cancer. Oncotarget. 2016;7:58162–73.
40. Chou FP, Chen YW, Zhao XF, Xu-Monette ZY, Young KH, Gartenhaus RB, et al. Imbalanced matriptase pericellular proteolysis contributes to the pathogenesis of malignant B-cell lymphomas. Am J Pathol. 2013;183:1306–17.
41. Leytus SP, Loeb KR, Hagen FS, Kurachi K, Davie EW. A novel trypsin-like serine protease (hepsin) with a putative transmembrane domain expressed by human liver and hepatoma cells. Biochemistry. 1988;27:1067–74.
42. Torres-Rosado A, O'Shea KS, Tsuji A, Chou SH, Kurachi K. Hepsin, a putative cell-surface serine protease, is required for mammalian cell growth. Proc Natl Acad Sci U S A. 1993;90:7181–5.
43. Yu IS, Chen HJ, Lee YS, Huang PH, Lin SR, Tsai TW, et al. Mice deficient in hepsin, a serine protease, exhibit normal embryogenesis and unchanged hepatocyte regeneration ability. Thromb Haemost. 2000;84:865–70.
44. Guipponi M, Tan J, Cannon PZF, Donley L, Crewther P, Clarke M, et al. Mice deficient for the type II transmembrane serine protease, TMPRSS1/hepsin, exhibit profound hearing loss. Am J Pathol. 2007;171:608–16.
45. Klezovitch O, Chevillet J, Mirosevich J, Roberts RL, Matusik RJ, Vasioukhin V. Hepsin promotes prostate cancer progression and metastasis. Cancer Cell. 2004;6:185–95.

46. Li W, Wang B-E, Moran P, Lipari T, Ganesan R, Corpuz R, et al. Pegylated kunitz domain inhibitor suppresses hepsin-mediated invasive tumor growth and metastasis. Cancer Res. 2009;69:8395–402.
47. Herter S, Piper DE, Aaron W, Gabriele T, Cutler G, Cao P, et al. Hepatocyte growth factor is a preferred in vitro substrate for human hepsin, a membrane-anchored serine protease implicated in prostate and ovarian cancers. Biochem J. 2005;390:125–36.
48. Betsunoh H, Mukai S, Akiyama Y, Fukushima T, Minamiguchi N, Hasui Y, et al. Clinical relevance of hepsin and hepatocyte growth factor activator inhibitor type 2 expression in renal cell carcinoma. Cancer Sci. 2007;98:491–8.
49. Hurst NJ, Najy AJ, Ustach CV, Movilla L, Kim H-RC. Platelet-derived growth factor-C (PDGF-C) activation by serine proteases: implications for breast cancer progression. Biochem J. 2012;441:909–18.
50. Ustach CV, Huang W, Conley-LaComb MK, Lin C-Y, Che M, Abrams J, et al. A novel signaling axis of matriptase/PDGF-D/ß-PDGFR in human prostate cancer. Cancer Res. 2010;70:9631–40.
51. Huang W, Kim H-RC. Dynamic regulation of platelet-derived growth factor D (PDGF-D) activity and extracellular spatial distribution by matriptase-mediated proteolysis. J Biol Chem. 2015;290:9162–70.
52. Najy AJ, Dyson G, Jena BP, Lin C-Y, Kim H-RC. Matriptase activation and shedding through PDGF-D-mediated extracellular acidosis. Am J Physiol Cell Physiol. 2016;310:C293–304.
53. Camerer E, Barker A, Duong DN, Ganesan R, Kataoka H, Cornelissen I, et al. Local protease signaling contributes to neural tube closure in the mouse embryo. Dev Cell. 2010;18:25–38.
54. Le Gall SM, Szabo R, Lee M, Kirchhofer D, Craik CS, Bugge TH, et al. Matriptase activation connects tissue factor-dependent coagulation initiation to epithelial proteolysis and signaling. Blood. 2016;127:3260–9.
55. Kanemaru A, Yamamoto K, Kawaguchi M, Fukushima T, Lin C-Y, Johnson MD, et al. Deregulated matriptase activity in oral squamous cell carcinoma promotes the infiltration of cancer-associated fibroblasts by paracrine activation of protease-activated receptor 2. Int J Cancer. 2017;140:130–41.
56. Kilpatrick LM, Harris RL, Owen KA, Bass R, Ghorayeb C, Bar-Or A, et al. Initiation of plasminogen activation on the surface of monocytes expressing the type II transmembrane serine protease matriptase. Blood. 2006;108:2616–23.
57. Moran P, Li W, Fan B, Vij R, Eigenbrot C, Kirchhofer D. Pro-urokinase-type plasminogen activator is a substrate for hepsin. J Biol Chem. 2006;281:30439–46.
58. Shimomura T, Denda K, Kitamura A, Kawaguchi T, Kito M, Kondo J, et al. Hepatocyte growth factor activator inhibitor, a novel Kunitz-type serine protease inhibitor. J Biol Chem. 1997;272:6370–6.
59. Kataoka H, Shimomura T, Kawaguchi T, Hamasuna R, Itoh H, Kitamura N, et al. Hepatocyte growth factor activator inhibitor type 1 is a specific cell surface binding protein of hepatocyte growth factor activator (HGFA) and regulates HGFA activity in the pericellular microenvironment. J Biol Chem. 2000;275:40453–62.
60. Kawaguchi T, Qin L, Shimomura T, Kondo J, Matsumoto K, Denda K, et al. Purification and cloning of hepatocyte growth factor activator inhibitor type 2, a Kunitz-type serine protease inhibitor. J Biol Chem. 1997;272:27558–64.
61. Delaria KA, Muller DK, Marlor CW, Brown JE, Das RC, Roczniak SO, et al. Characterization of placental bikunin, a novel human serine protease inhibitor. J Biol Chem. 1997;272:12209–14.
62. Kataoka H, Suganuma T, Shimomura T, Itoh H, Kitamura N, Nabeshima K, et al. Distribution of hepatocyte growth factor activator inhibitor type 1 (HAI-1) in human tissues. Cellular surface localization of HAI-1 in simple columnar epithelium and its modulated expression in injured and regenerative tissues. J Histochem Cytochem. 1999;47:673–82.
63. Kataoka H, Meng JY, Itoh H, Hamasuna R, Shimomura T, Suganuma T, et al. Localization of hepatocyte growth factor activator inhibitor type 1 in Langhans' cells of human placenta. Histochem Cell Biol. 2000;114:469–75.

64. Tanaka H, Nagaike K, Takeda N, Itoh H, Kohama K, Fukushima T, et al. Hepatocyte growth factor activator inhibitor type 1 (HAI-1) is required for branching morphogenesis in the chorioallantoic placenta. Mol Cell Biol. 2005;25:5687–98.
65. Mukai S, Fukushima T, Naka D, Tanaka H, Osada Y, Kataoka H. Activation of hepatocyte growth factor activator zymogen (pro-HGFA) by human kallikrein 1-related peptidases. FEBS J. 2008;275:1003–17.
66. Mukai S, Yorita K, Yamasaki K, Nagai T, Kamibeppu T, Sugie S, et al. Expression of human kallikrein 1-related peptidase 4 (KLK4) and MET phosphorylation in prostate cancer tissue: immunohistochemical analysis. Hum Cell. 2015;28:133–42.
67. Lin CY, Anders J, Johnson M, Dickson RB. Purification and characterization of a complex containing matriptase and a Kunitz-type serine protease inhibitor from human milk. J Biol Chem. 1999;274:18237–42.
68. Lai C-H, Lai Y-JJ, Chou F-P, Chang H-HD, Tseng C-C, Johnson MD, et al. Matriptase complexes and prostasin complexes with HAI-1 and HAI-2 in human milk: significant proteolysis in lactation. PLoS One. 2016;11:e0152904.
69. Kohama K, Kawaguchi M, Fukushima T, Lin C-Y, Kataoka H. Regulation of pericellular proteolysis by hepatocyte growth factor activator inhibitor type 1 (HAI-1) in trophoblast cells. Hum Cell. 2012;25:100–10.
70. Kawaguchi M, Kanemaru A, Sawaguchi A, Yamamoto K, Baba T, Lin C, et al. Hepatocyte growth factor activator inhibitor type 1 maintains the assembly of keratin into desmosomes in keratinocytes by regulating protease-activated receptor 2-dependent p38 signaling. Am J Pathol. 2015;185:1610–23.
71. Baba T, Kawaguchi M, Fukushima T, Sato Y, Orikawa H, Yorita K, et al. Loss of membrane-bound serine protease inhibitor HAI-1 induces oral squamous cell carcinoma cells' invasiveness. J Pathol. 2012;228:181–92.
72. Fukushima T, Kawaguchi M, Yamasaki M, Tanaka H, Yorita K, Kataoka H. Hepatocyte growth factor activator inhibitor type 1 suppresses metastatic pulmonary colonization of pancreatic carcinoma cells. Cancer Sci. 2011;102:407–13.
73. Ye J, Kawaguchi M, Haruyama Y, Kanemaru A, Fukushima T, Yamamoto K, et al. Loss of hepatocyte growth factor activator inhibitor type 1 participates in metastatic spreading of human pancreatic cancer cells in a mouse orthotopic transplantation model. Cancer Sci. 2014;105:44–51.
74. Hoshiko S, Kawaguchi M, Fukushima T, Haruyama Y, Yorita K, Tanaka H, et al. Hepatocyte growth factor activator inhibitor type 1 is a suppressor of intestinal tumorigenesis. Cancer Res. 2013;73:2659–70.
75. Kawaguchi M, Yamamoto K, Kanemaru A, Tanaka H, Umezawa K, Fukushima T, et al. Inhibition of nuclear factor-κB signaling suppresses Spint1-deletion-induced tumor susceptibility in the ApcMin/+ mouse model. Oncotarget. 2016;7:68614–22.
76. Kataoka H, Uchino H, Denda K, Kitamura N, Itoh H, Tsubouchi H, et al. Evaluation of hepatocyte growth factor activator inhibitor expression in normal and malignant colonic mucosa. Cancer Lett. 1998;128:219–27.
77. Heinz-Erian P, Müller T, Krabichler B, Schranz M, Becker C, Rüschendorf F, et al. Mutations in SPINT2 cause a syndromic form of congenital sodium diarrhea. Am J Hum Genet. 2009;84:188–96.
78. Friis S, Sales KU, Schafer JM, Vogel LK, Kataoka H, Bugge TH. The protease inhibitor HAI-2, but not HAI-1, regulates matriptase activation and shedding through prostasin. J Biol Chem. 2014;289:22319–32.
79. Fukai K, Yokosuka O, Chiba T, Hirasawa Y, Tada M, Imazeki F, et al. Hepatocyte growth factor activator inhibitor 2/placental bikunin (HAI-2/PB) gene is frequently hypermethylated in human hepatocellular carcinoma. Cancer Res. 2003;63:8674–9.
80. Yamauchi M, Kataoka H, Itoh H, Seguchi T, Hasui Y, Osada Y. Hepatocyte growth factor activator inhibitor types 1 and 2 are expressed by tubular epithelium in kidney and down-regulated in renal cell carcinoma. J Urol. 2004;171:890–6.

81. Morris MR, Gentle D, Abdulrahman M, Clarke N, Brown M, Kishida T, et al. Functional epigenomics approach to identify methylated candidate tumour suppressor genes in renal cell carcinoma. Br J Cancer. 2008;98:496–501.
82. Hamasuna R, Kataoka H, Meng JY, Itoh H, Moriyama T, Wakisaka S, et al. Reduced expression of hepatocyte growth factor activator inhibitor type-2/placental bikunin (HAI-2/PB) in human glioblastomas: implication for anti-invasive role of HAI-2/PB in glioblastoma cells. Int J Cancer. 2001;93:339–45.
83. Kongkham PN, Northcott PA, Ra YS, Nakahara Y, Mainprize TG, Croul SE, et al. An epigenetic genome-wide screen identifies SPINT2 as a novel tumor suppressor gene in pediatric medulloblastoma. Cancer Res. 2008;68:9945–53.
84. Hwang S, Kim H-E, Min M, Raghunathan R, Panova IP, Munshi R, et al. Epigenetic silencing of *SPINT2* promotes cancer cell motility via HGF-MET pathway activation in melanoma. J Invest Dermatol. 2015;135:2283–91.
85. Müller-Pillasch F, Wallrapp C, Bartels K, Varga G, Friess H, Büchler M, et al. Cloning of a new Kunitz-type protease inhibitor with a putative transmembrane domain overexpressed in pancreatic cancer. Biochim Biophys Acta. 1998;1395:88–95.
86. Pereira MS, de Almeida GC, Pinto F, Viana-Pereira M, Reis RM. SPINT2 deregulation in prostate carcinoma. J Histochem Cytochem. 2016;64:32–41.
87. Tsai CH, Teng CH, YT T, Cheng TS, SR W, Ko CJ, et al. HAI-2 suppresses the invasive growth and metastasis of prostate cancer through regulation of matriptase. Oncogene. 2014;33:4643–52.
88. Ganesan R, Eigenbrot C, Kirchhofer D. Structural and mechanistic insight into how antibodies inhibit serine proteases. Biochem J. 2010;430:179–89.
89. Eigenbrot C, Ganesan R, Kirchhofer D. Hepatocyte growth factor activator (HGFA): molecular structure and interactions with HGFA inhibitor-1 (HAI-1). FEBS J. 2010;277:2215–22.
90. Han Z, Harris PKW, Karmakar P, Kim T, Owusu BY, Wildman SA, et al. α-Ketobenzothiazole serine protease inhibitors of aberrant HGF/c-MET and MSP/RON kinase pathway signaling in cancer. ChemMedChem. 2016;11:585–99.
91. Venukadasula PKM, Owusu BY, Bansal N, Ross LJ, Hobrath JV, Bao D, et al. Design and synthesis of nonpeptide inhibitors of hepatocyte growth factor activation. ACS Med Chem Lett. 2016;7:177–81.
92. Han Z, Harris PKW, Jones DE, Chugani R, Kim T, Agarwal M, et al. Inhibitors of HGFA, matriptase, and hepsin serine proteases: a nonkinase strategy to block cell signaling in cancer. ACS Med Chem Lett. 2014;5:1219–24.
93. Owusu BY, Bansal N, Venukadasula PKM, Ross LJ, Messick TE, Goel S, et al. Inhibition of pro-HGF activation by SRI31215, a novel approach to block oncogenic HGF/MET signaling. Oncotarget. 2016;7:29492–506.

Chapter 10
Molecular Designing of Small-Molecule Inhibitors for Apoptosis Regulation

Atsushi Yoshimori and Sei-Ichi Tanuma

Abstract Apoptosis is a distinctive mode of programmed cell death, which is involved in organ life cycle in multicellular organism. Dysregulation of apoptotic processes has been implicated in a wide variety of diseases, such as cancer, neurodegenerative disorders, and ischemic injury. To date, many kinds of key proteins in apoptotic processes have been identified and targeted for therapeutic strategies. Several effective small molecules have been designed to modulate the key regulatory proteins, such as Bcl-2, XIAP, MDM2, and caspases. This chapter reviews the current development of small-molecule inhibitors targeting apoptosis regulatory proteins, and as an example, our structure-based approaches for the designing of caspase-3-specific inhibitors will be described.

Keywords Apoptosis • Protein-protein interactions • Structure-based drug design • Small molecules

10.1 Introduction

Apoptosis is a process of programmed cell death, which enables to eliminate damaged and harmful cells to maintain homeostatic cellular balances in multicellular organism [1, 2]. The processes of apoptosis are typically characterized by morphological features, such as cell shrinkage, nuclear condensation, and apoptotic body formation [3, 4]. A biochemical hallmark of apoptosis is internucleosomal DNA fragmentation [5]. Apoptosis is stimulated by exogenous and endogenous factors, including oxidative stress, ultraviolet radiation, and viral infection [6–8]. These stimuli activate apoptotic pathway, which leads to remove abnormal cells in organs.

A. Yoshimori (✉)
Institute for Theoretical Medicine, Inc., Yokohama, Japan
e-mail: yoshimori@itmol.com

S.-I. Tanuma
Department of Biochemistry, Faculty of Pharmaceutical Sciences, Tokyo University of Science, Chiba, Japan

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N. Shinomiya et al. (eds.), *Regulation of Signal Transduction in Human Cell Research*, Current Human Cell Research and Applications,
https://doi.org/10.1007/978-981-10-7296-3_10

Since apoptosis is a highly controlled process of cell elimination and plays a fundamental role in tissue homeostasis, dysregulation of apoptotic signalings causes many human diseases [9].

Abnormal activation of apoptosis causes neurodegenerative disorders, liver injury, viral infection, and so on [8, 10, 11]. For example, increased hepatocyte apoptosis contributes to liver injury and fibrosis. IDN-6556 (pan-caspase inhibitor) decreased liver injury and fibrosis in a murine model of nonalcoholic steatohepatitis [12]. Caspases are a family of cysteine protease, which are one of the executors of apoptotic processes [13]. IDN-6556 has been used in clinical trials for the treatment of a number of hepatic diseases [14]. Meanwhile, suppression of apoptosis causes cancer, premalignant diseases, metabolic disorders, and so on [15–17]. One of the hallmarks of human cancers is the resistance to apoptosis [18]. Thus, modulation of the apoptotic signalings has been proposed as a new strategy for cancer therapy, and many drug targets have been identified for the development of anticancer drugs [19]. For example, inhibitor of apoptosis family proteins (IAPs) have been shown to promote pro-survival signaling and inhibit apoptotic signaling cascades [20]. Notably, X-linked IAP (XIAP) interacts directly with caspase-3, caspase-7, and caspase-9 and interferes with the activation of the caspases [21, 22]. AT-401 (IAP antagonist) effectively inhibits tumor growth in a MDA-MB-231 xenograft model and is in clinical trials for a treatment of human cancer [23].

To date, many kinds of key proteins in apoptotic signalings have been identified and targeted for the discovery of pharmaceuticals for apoptotic diseases, such as cancers and neurodegenerative disorders [19]. Moreover, X-ray crystal structures of their key proteins have been solved during the course of both basic science and structure-based drug design researches [24–27]. This chapter reviews mainly the current development of small-molecule inhibitors designed using peptide mimetics and X-ray crystal structures to modulate apoptotic signalings and describes our structure-based drug design approaches aimed at the development of caspase-3-specific inhibitors.

10.2 Drug Targets in Apoptotic Pathways

Apoptosis signalings are introduced through death receptor-mediated and mitochondrial-mediated pathways, which are known as extrinsic and intrinsic pathways, respectively (Fig. 10.1) [28]. The extrinsic pathway of apoptosis is activated by death receptors on cell surface, including TNF receptor-1, Fas, TRAIL receptor 1 (TRAIL-R1), and TRAIL receptor 2 (TRAIL-R2, DR5) [29–31]. Upon binding their cognate ligands such as tumor necrosis factor, Fas ligand, and TRAIL, the death receptors aggregate and form death-inducing signaling complex (DISC) in their intracellular regions [32]. Then, procaspase-8 is activated by forming of the DISC, and activated caspase-8 cleaves and activates downstream executioner caspases, such as caspase-3 and caspase-7 [33, 34]. The executioner caspases cleave a number of protein substrates, such as ICAD, PARP, and Acinus, leading to internucleosomal DNA fragmentation [35–38]. Moreover, caspase-8 cleaves Bid for cross

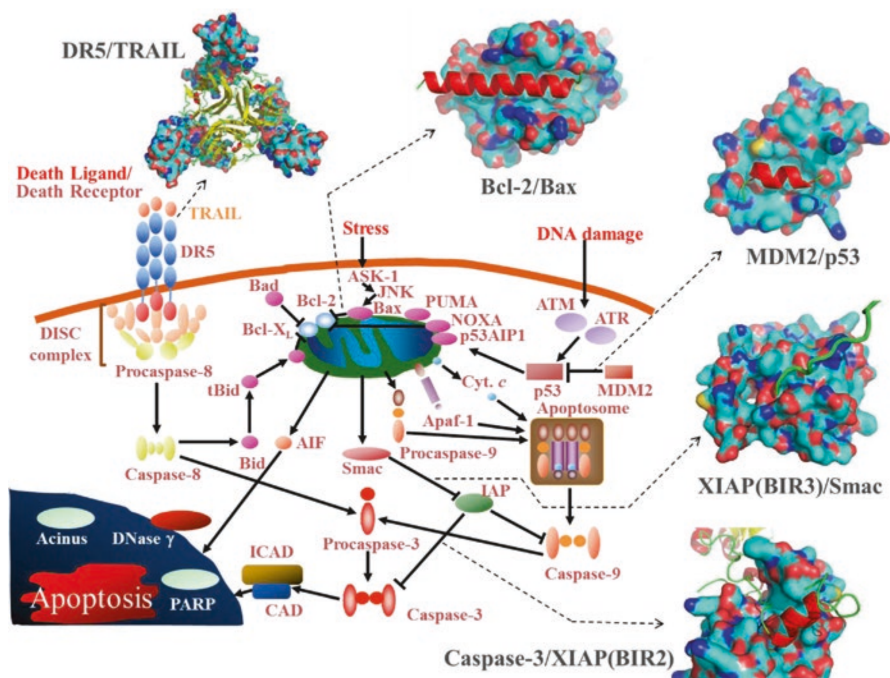


Fig. 10.1 Apoptotic signaling pathway and examples of drug targets for structure-based drug design. The crystal structures of the drug targets are DR5/TRAIL (PDB entry 1D0G), Bcl-2/Bax (PDB entry 2XA0), MDM2/p53 (PDB entry 4HFZ), XIAP (BIR3)/Smac (PDB entry 1G73), and caspase-3/XIAP(BIR2) (PDB entry 1I3O). The molecular surface representation shows DR5, Bcl-2, MDM2, XIAP (BIR3), and caspase-3. The ribbon diagram shows TRAIL, Bax, p53, Smac, and XIAP (BIR2)

talk to the intrinsic pathway [39]. In order to induce apoptosis against various cancer cells, agonistic TRAIL-R1 and TRAIL-R2 monoclonal antibodies have been developed for clinical cancer therapy [40]. Moreover, small molecules as TRAIL mimetics targeting DR5 have been discovered through high-throughput chemical screenings [41].

The intrinsic pathway is triggered by signals, such as reactive oxygen species (ROS), ER stress, and DNA damage, that induce the release of pro-apoptotic factors, such as cytochrome c, from mitochondria into the cytosol [42]. This leads to formation of apoptosome, which subsequently induces the activation of procaspase-9 [43]. Activated caspase-9 cleaves the downstream executioner caspases, leading to internucleosomal DNA fragmentation by the mechanism common to the extrinsic pathway. Bcl-2 family proteins have critical roles in the intrinsic pathway by modulating mitochondrial membrane permeability and the release of pro-apoptotic factors [44]. They classify into pro-apoptotic (Bax, Bak, Bid, Bim, Bad, and Noxa) and anti-apoptotic (Bcl-2, Bcl-xL, and Mcl-1) proteins [45]. The anti-apoptotic Bcl-2 family proteins are frequently overexpressed in cancers [46]. For example, Bcl-2 directly binds to Bax via Bcl-2 homology (BH) 1–3 groove-BH3 domain interface and inhibits apoptosis [47]. Thus, several small molecules have been synthesized as

Bcl-2 antagonists for cancer therapy [48]. IAPs are a family of proteins containing several baculovirus IAP repeat (BIR) domains [20]. X-linked IAP (XIAP) is one of the most characterized of IAPs. The BIR2 domain of XIAP binds to caspase-3 and caspase-7, while the BIR3 domain binds to caspase-9. These interactions inhibit the activity of the caspases and interfere with apoptosis [21, 22]. The interaction between XIAP and caspases is interfered by second mitochondria-derived activator of caspase (Smac), which is released from mitochondria and promotes caspase activation [49]. On the basis of this role, several small-molecule Smac mimetics (XIAP antagonists) are in clinical trials for cancer treatment [50]. Mouse double minute 2 homolog (MDM2) regulates tumor suppressor p53 levels by promoting p53 ubiquitination and thereby proteasome-mediated degradation [51]. Therefore, the inhibition of MDM2/p53 interaction has been proposed as a new approach for the treatment of cancer [52]. Over the past decades, apoptosis-based therapies have attracted attention from pharmaceutical companies.

10.3 Small Molecular Modulators of Apoptosis

In the apoptotic signalings, protein-protein interactions (PPIs) are fundamental events in both extrinsic and intrinsic pathways [28]. Small molecular modulation of the PPIs provides an attractive approach for drug discovery against dysregulated apoptotic processes. Inhibitors (or antagonists) of the PPIs, such as Bcl-2/Bax, MDM2/p53, and XIAP (BIR3)/Smac, are representative examples of small molecular modulators of apoptosis. Here, we briefly review the small molecular modulators of apoptosis.

10.3.1 Antagonists of Bcl-2 Family Proteins

The anti-apoptotic Bcl-2 proteins and Bak/Bax share four conserved domains, known as BH1, BH2, BH3, and BH4. The pro-apoptotic Bcl-2 proteins, such as Bid, Bim, Bad, and Noxa, share only the BH3 domain (BH3-only proteins) [45]. The BH1–3 domains form a hydrophobic groove on the surface of the anti-apoptotic proteins, which interacts with the BH3 domain of its pro-apoptotic partner proteins [47]. BH3-derived peptides from pro-apoptotic proteins bind to the hydrophobic groove of anti-apoptotic proteins and induce apoptosis [53]. Structurally, the BH3-derived peptides form α-helical structures [54]. Thus, small molecules which mimic the BH3 domain have been developed as modulators of apoptosis.

α-helix mimetics are expected to bind to the anti-apoptotic proteins by mimicking the BH3 domain. A terphenyl scaffold was used as template to mimic the α-helix structure of the BH3 domain. The terphenyl derivative (Fig. 10.2a) reproduced the spatial arrangement of amino acids at the i, $i + 4$, and $i + 7$ positions in the α-helix and has a K_d value for Bcl-xL of 114 nM [55]. Oligoamide-based α-helix mimetics led to the discovery of the JY-1-106 (K_i for Bcl-xL = 179 nM) (Fig. 10.2b) [56].

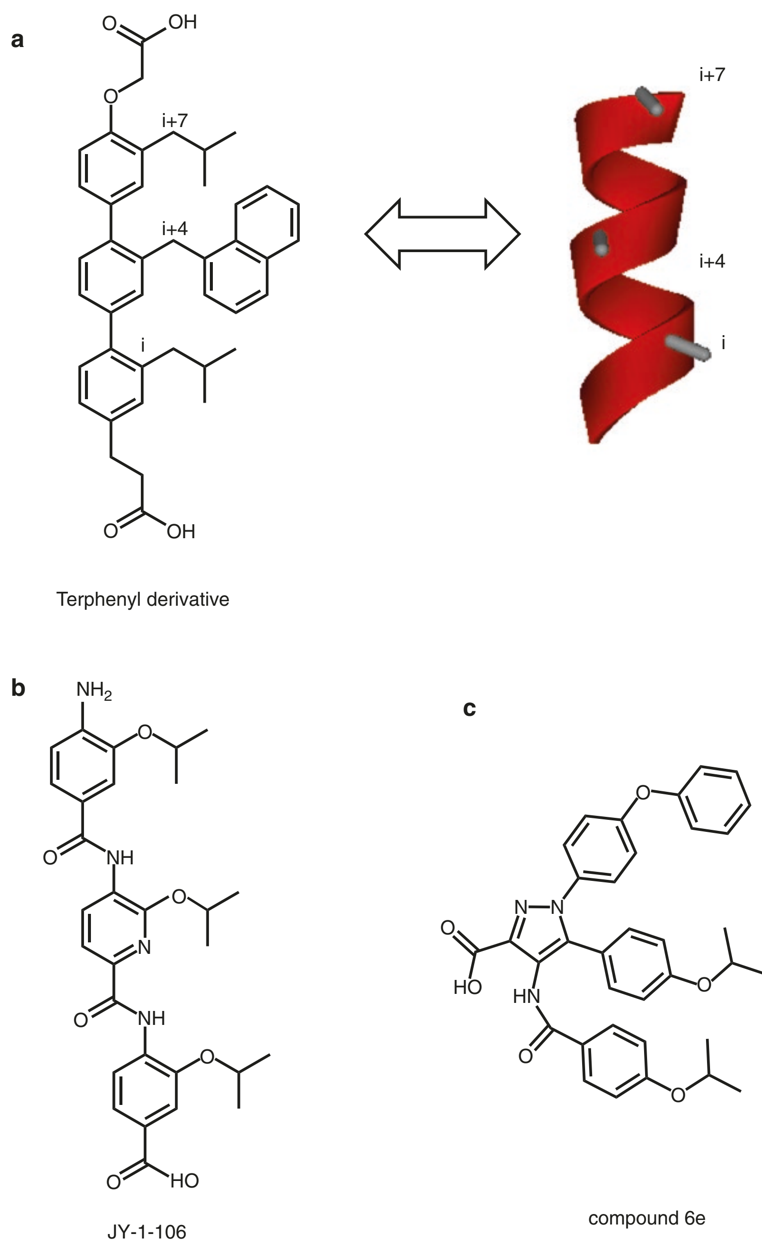


Fig. 10.2 α-helix mimetics for Bcl-2 family antagonists. (**a**) Terphenyl derivative (*left side*) and α-helix (*right side*), with the *i*, *i* + 4, and *i* + 7 position being represented by *gray* stick model. (**b**) Oligoamide derivative (JY-1-106). (**c**) Pyramid-like α-helix mimetic (compound 6e)

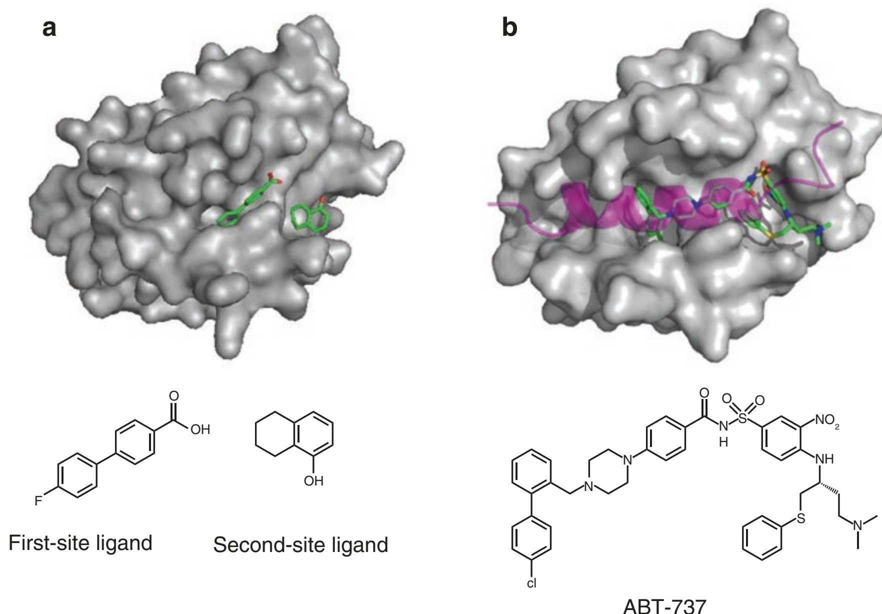


Fig. 10.3 Application of the SAR by NMR for design of Bcl-2 family antagonists. (**a**) First- and second-site ligands discovered by nuclear magnetic resonance-based screening (PDB entry 1YSG). The two ligands are represented in stick model. Bcl-xL is shown in *gray* surface representation. (**b**) Superposition of Bcl-xL/ABT-737 complex (PDB entry 2YXJ) and Bcl-xL/Bad complex (PDB entry 1G5J). ABT-737 is represented in stick model. BCL-xL is shown in *gray* surface. Bad is shown in *magenta ribbon* representation

Recently, a pyramid-like scaffold was proposed as universal α-helical mimetics. Compound 6e based on the pyramid-like scaffold was reported as Bcl-2/MDM2 dual inhibitors (Fig. 10.2c) [57].

Employing nuclear magnetic resonance (NMR)-based screening and parallel synthesis, small-molecule antagonists have been designed to mimic the binding of the BH3 domain to anti-apoptotic proteins [58]. The strategy was named by SAR by NMR, which is now widely used in drug discovery. First, a fluoro-biaryl acid as a first-site ligand was screened by the NMR-based screening (Fig. 10.3a). In NMR-based titration experiments, this compound has a K_d value for Bcl-xL of ~300 μM. Next, several naphthol analogues and a biaryl phenol as second-site ligands were identified that bound to the site in the presence of the fluoro-biaryl acid (Fig. 10.3a). And then, the first- and second-site ligands were connected by various linkers. Finally, a high-affinity ligand was obtained, which binds to Bcl-xL with K_i of 36 nM [58]. From SAR by NMR, ABT-737 has been designed to mimic the binding of the BH3 domain to anti-apoptotic proteins (K_i for Bcl-2 and Bcl-xL < 1 nM) (Fig. 10.3b) [48, 59].

Table 10.1 IAP-binding motif

IAP-binding protein	Sequence
Smac	**A V P I** A Q K S
Reaper	**A V A F** Y I P D
Hid	**A V P F** Y L P E
Grim	**A I A Y** F I P D
Sickle	**A I P F** F E E E

10.3.2 Antagonists of IAP Proteins

IAPs, including cIAP1, cIAP2, XIAP, and ML-IAP, comprise a family of anti-apoptosis proteins that repress apoptosis progression by interfering with the activation of caspases in either a direct or indirect manner [60]. Overexpression of IAPs occurs in many cancers and promotes resistance to chemotherapy and radiation [61]. XIAP is the best characterized IAPs. The BIR2 domain of XIAP binds to caspase-3 and caspase-7, while the BIR3 domain binds to caspase-9. These direct interactions suppress the activation of the caspases and inhibit apoptosis [21, 22]. An endogenous inhibitor of IAPs, called Smac, is a pro-apoptotic protein that is released from mitochondria in response to apoptotic stimuli into the cytosol and competes with the caspases for binding to several IAPs and thereby facilitates apoptosis [49]. Smac interacts with IAPs via its N-terminal amino acid residues (AVPI), named IAP-binding motif (IBM). An alignment of IAP-binding proteins provided the sequence motif of A-(V/I)-(P/A)-(F/I/Y) (Table 10.1) [62]. A 4-mer peptide (AVPI) derived from Smac has the ability to inhibit Smac-BIR3 interaction (IC_{50} = 0.58 μM). Moreover, AVPF was found to have a higher inhibitory activity (IC_{50} = 0.12 μM) than AVPI [62]. Phe in the fourth position in AVPF tightly interacts with the BIR3 pocket of XIAP (Fig. 10.4a). However, although the 4-mer peptides have potent inhibitory activity, they do not have good pharmacological property. Therefore, small-molecule IBM mimetics have been developed. GDC-0152 was designed as antagonist of IAPs from the IBM, which binds to the BIR3 domain of cIAP1, cIAP2, and XIAP and the BIR domain of ML-IAP with K_i values of 17, 43, 28, and 14 nM, respectively [63]. IBMs are attractive starting point for designing of IAP antagonists. GDC-0152 is in currently being tested in clinical trials as novel cancer therapeutics [50]. IAP antagonists can be divided into monovalent and bivalent antagonists. Examples for monovalent IAP antagonists are GDC-0152, AT-406, MV1, and LBW242 (Fig. 10.4b) [63–66]. Bivalent inhibitors, for example, are BV6 and TL-32711, which consist of two monovalent antagonist that are connected via a linker (Fig. 10.4c) [65, 67]. Bivalent antagonists generally demonstrate higher binding affinity and better cellular potencies than monovalent antagonists [65]. Recently, macrocyclic XIAP antagonists have been screened from a DNA-programmed chemistry library [68]. The macrocyclic derivative (compound 18) has IC_{50} values 97 nM against XIAP-BIR2, 36 nM against XIAP-BIR3, and 16 nM against cIAP1-BIR3 (Fig. 10.4d) [68].

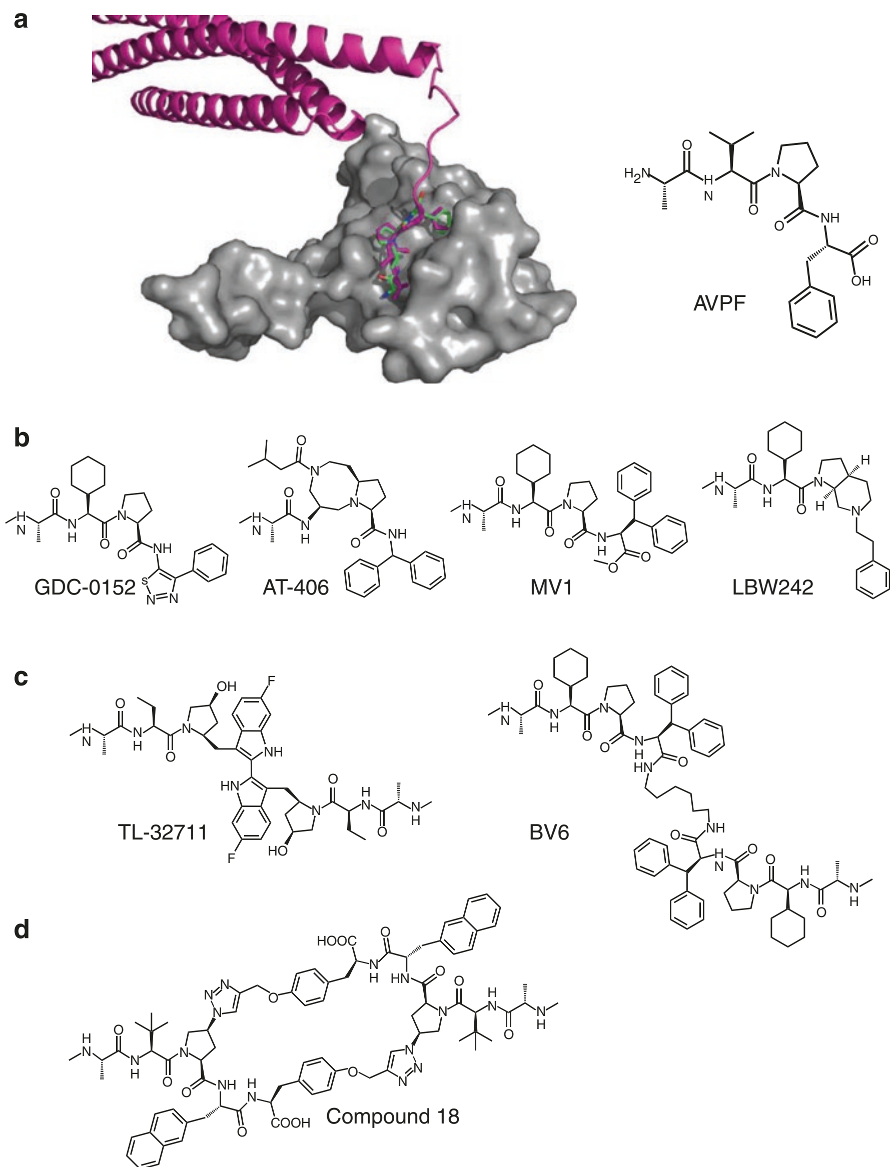
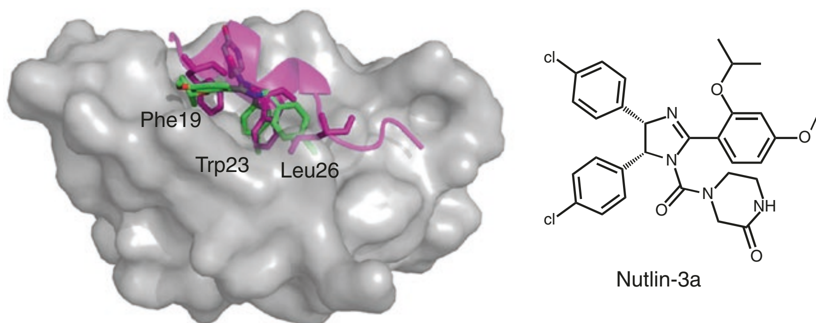
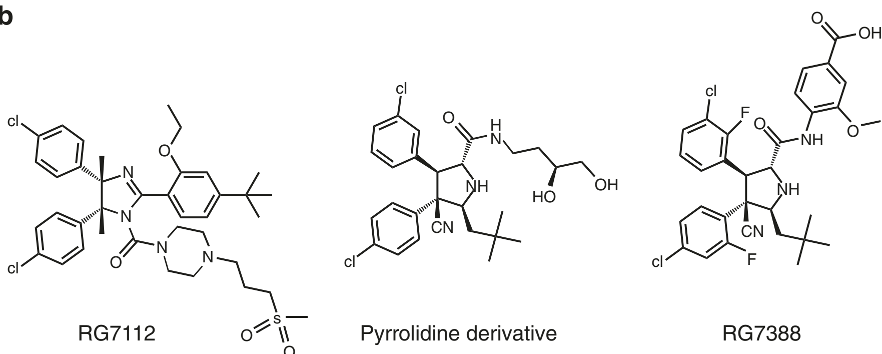


Fig. 10.4 IAP antagonists. (**a**) Superposition of XIAP/Smac complex (PDB entry 1G73) and XIAP/AVPF complex (PDB entry 2OPZ). AVPF is represented in stick model with carbon colored *green*, oxygen colored *red*, and nitrogen colored *blue*. Smac is shown in *magenta ribbon* representation, and its N-terminal AVPI motif is shown in *magenta stick* representation. XIAP is shown in *gray* surface representation. (**b**) Monovalent antagonists. (**c**) Bivalent antagonist. (**d**) Macrocyclic antagonist

10.3.3 Inhibitors of MDM2

The tumor suppressor protein p53 is a transcriptional factor that plays vital roles in the regulation of several cellular processes [69]. In approximately 50% of human cancers, p53 is mutated or deleted [70]. Meanwhile, in human cancers retaining wild-type p53, the mouse double minute 2 (MDM2; HDM2 in humans) binds to the N-terminal region of p53 and inhibits its function in cells [51]. The interfering with the protein-protein interaction of MDM2 and p53 would release p53 from MDM2 and reactivate its tumor suppressor function. Thus, small-molecule inhibitors (MDM inhibitors) may have a therapeutic benefit in human cancers with wild-type p53. The N-terminal region of p53 interacts with MDM2 in an α-helix conformation, and the most important residues in the region are three hydrophobic residues, Phe19, Trp23, and Leu26 (Fig. 10.5a) [52]. Nutlins are first reported as the potent and specific MDM2 inhibitors, which are *cis*-imidazoline derivatives [71]. The initial lead compounds were discovered by screening of a chemical library. One of the most promising inhibitors in nutlin is nutlin-3a, which inhibits the MDM2/p53 interaction, with a IC_{50} value of 90 nM [72]. Nutlins occupy the same pockets as the three hydrophobic residues (Phe19, Trp23, and Leu26) of p53 in MDM2 cleft (Fig. 10.5a) [72]. Additionally, RG7112 was developed based on the structure-based optimization of nutlin-3a. RG7112 is in clinical trials for treatment of human cancers [73]. The most representative of MDM2 inhibitors is shown in Fig. 10.5b. RG7388 is a second-generation MDM2 inhibitor, which possesses pyrrolidine scaffold. The stereochemical configuration of the pyrrolidine scaffold in which the two phenyl groups adopt a *trans* configuration was very important for potent inhibitory effects (IC_{50} = 6 nM) [74]. The crystal structure of MDM/pyrrolidine derivative (Fig. 10.5b) complex (PDB entry 4JRG) shows that 2-chloropheny group of pyrrolidine derivative occupies the Leu26 pocket and establishes π−π stacking interaction with His96 of MDM2 in addition to mimicking the three hydrophobic residues of p53 [74]. The additional interactions not observed between p53 and MDM2 could be important for designing potent MDM2 inhibitors. Another negative regulator of p53 is MDMX, which may directly regulate p53 transcription [75]. Although nutlin-3a binds to MDMX, its binding affinity is >200-fold less potent than that toward MDM2 [76]. Several MDMX inhibitors were reported (Fig. 10.5c). SJ-172550 was discovered as the first MDMX inhibitor by high-throughput screening of chemical libraries [77]. The binding of SJ-172550 to MDMX forms both covalent and non-covalent interactions (IC_{50} = 3 μM) [78]. Indolyl hydantoin compounds were screened for suppression of p53/MDMX binding from a diverse library of small molecules. One of the compound (RO-2443) had inhibitory activities against both MDM2 (IC_{50} = 33 nM) and MDMX (IC_{50} = 41 nM). RO-2443 induces homo- and/or hetero-dimerization of MDM2 and MDMX and then blocks p53 binding with both MDM2 and MDMX [79].





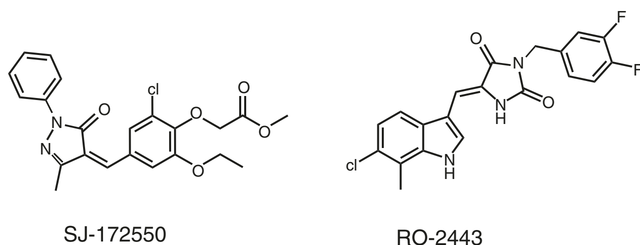


Fig. 10.5 MDM2/MDMX inhibitors. (**a**) Superposition of MDM2/p53 complex (PDB entry 1YCR) and MDM2/Nutlin-3a complex (PDB entry 4HG7). Nutlin-3a is represented in stick model with carbon colored *green*, oxygen colored *red*, and nitrogen colored *blue*. p53 is shown in *magenta ribbon* representation, and three hydrophobic residues (Phe19, Trp23, and Leu26) are shown in *magenta stick* representation. (**b**) MDM2 inhibitors. (**c**) MDMX inhibitors

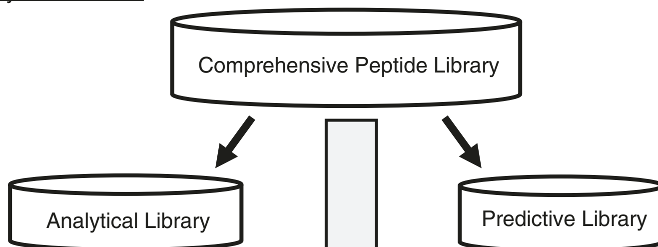
10.4 Molecular Design of Small-Molecule Inhibitors of Apoptosis-Related Proteins: A Case Study on Caspase-3-Specific Inhibitors

Caspases are a family of cysteine protease that play critical roles in apoptosis and inflammation. Apoptotic caspases are grouped into either initiator (caspase-2, caspase-8, caspase-9, and caspase-10) or executioner (caspase-3, caspase-6, and caspase-7) caspases. Autoactivation of initiator caspases is facilitated by adaptor proteins, such as apoptosis protease activating factor-1 (Apaf-1) and Fas-associated death domain protein (FADD). Executioner caspases are activated by cleavage from initiator caspases and then cleave hundreds of different substrate proteins, leading to cell death. Dysregulation of caspases causes a variety of diseases, including neurodegenerative disorders and cancers [13].

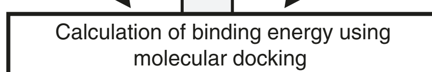
Caspases share similarities in amino acid sequences, structure, and substrate specificities. The three-dimensional structures show that the active sites of all caspases have positively charged S_1 subsites that interact with the negatively charged Asp in the P_1 position on the substrate proteins. Because the S_1 subsites on caspases are highly conserved, all caspases cleave substrate proteins in a specific manner after the Asp residue [27, 80]. Recognition of at least four amino acids (P_1-P_4) is also an essential requirement of efficient catalysis. The S_2–S_4 subsites on caspases vary significantly, resulting in various substrate specificities for the P_2–P_4 positions, in spite of an absolute requirement for Asp in the P_1 position. The sequence DEVD with in poly(ADP-ribose) polymerase (PARP) is known to be recognized [81] and cleaved by caspase-3, and it has been used to create a caspase-3 inhibitor Ac-DEVD-CHO. However, Ac-DEVD-CHO inhibits not only caspase-3 activity but also the activities of some other caspases [82]. Therefore, it could be difficult to design selective inhibitors based on the sequence of known cleavage sites.

To identify the sequence with the highest binding affinity against each caspase, the X-X-X-D, where “X” denotes any of 20 amino acids, sequence motif is used for structure-based virtual screening (SBVS), in which a total of 20^3 binding energy calculations are performed. For clearly showing each amino acid position, X-X-X-D motif is expressed as P_4-P_3-P_2-D. Generally, SBVS of a large library of compounds requires large-scale computations. Since, in particular, peptides are highly flexible with a number of rotational bonds, long computational time of binding energy calculation is required. Here, we introduce our new computational screening system based on Amino Acid Positional Fitness (APF) score for rapid calculation of binding affinity between a peptide and a target protein (Fig. 10.6) [83]. In our system, 1–10% of peptides in the comprehensive peptide library are selected at random. The binding energies between the selected peptides and the target protein are calculated using molecular docking software, where we use AutoDock 3.0 [84], and then the binding energies are transferred to APF score matrix using statistical technique. Ultimately, the APF score matrix is able to calculate rapidly the binding score (APF score) of all peptides in the comprehensive peptide library. We show here the usefulness of the APF score for design of potent and specific caspase-3 inhibitor.

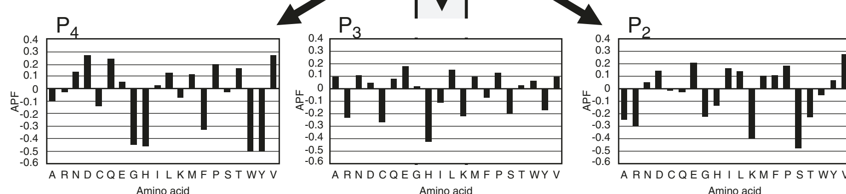
Step 1: Library Construction



Step 2: Binding Energy Calculation



Step 3: APF Score Matrix Construction



Step 4: Enriched Library Construction

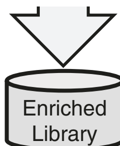


Fig. 10.6 General outline of the APF method

10.4.1 Construction of Screening System for Caspase-3 Inhibitors

The APF score matrix allows us to calculate binding affinities between peptides and a target protein. A general outline for constructing the APF score matrix for caspase-3 is illustrated in Fig. 10.6. This screening system is named APF method, and details are described as follows [83]:

10.4.1.1 Step 1: Construction of Peptide Libraries

To identify the P_2–P_4 preferences of caspase-3, we constructed the comprehensive peptide library, which contained 20^3 peptides with Asp fixed at P_1. The analytical library contains 360 peptides randomly selected from the comprehensive peptide library, and the predictive library contains 40 peptides randomly selected from the comprehensive peptide library.

10.4.1.2 Step 2: Calculation of Binding Energies Using Molecular Docking Software

The binding energies between 400 peptides contained in the analytical and predictive library and caspase-3 were calculated by using molecular docking software AutoDock 3.0. The crystal structure of caspase-3 was obtained from Protein Data Bank (PDB entry 1PAU).

10.4.1.3 Step 3: Construction of APF Score Matrix

APF score matrix was generated based on the frequency of appearance of 20 amino acids at each position (P_4, P_3, and P_2) of peptides in the analytical library. The APF score of amino acid i at position j is calculated as follows:

$$\mathrm{APF}_{ij} = \log\left(\frac{\rho_{\mathrm{binder}}^{ij}}{\rho_{\mathrm{all}}^{ij}}\right) \tag{10.1}$$

$\rho_{\mathrm{all}}{}^{ij}$ is the frequency of amino acid i at position j among peptides in the analytical library, and $\rho_{\mathrm{binder}}{}^{ij}$ is the frequency of amino acid i at position j among peptides below a threshold value of binding energy.

$$\rho_{\mathrm{all}}^{ij} = \frac{c_{ij}}{n_{\mathrm{all}}} \tag{10.2}$$

$$\rho_{\mathrm{binder}}^{ij} = \frac{c'_{ij}}{n_{\mathrm{binder}}} \tag{10.3}$$

c_{ij} denotes the number of times that amino acid i appears at position j among peptides in the analytical library. n_{all} is the number of peptides in the analytical library. c_{ij}' denotes the number of times that amino acid i appears at position j among peptides below the threshold value of binding energy in the analytical library. n_{binder} is the number of peptides below the threshold value of binding energy in the analytical library. The range of position j is from one to three and corresponding to P_2 to P_4. The range of amino acid i is from 1 to 20 and corresponding to individual amino acids.

APF score matrix allows us to calculate the APF score, which indicates the binding affinity between a peptide and caspase-3. The APF score of a peptide is calculated as follows:

$$\mathrm{APF\,score} = \sum_{i=1}^{20}\sum_{j=1}^{3} c_{ij}\mathrm{APF}_{ij} \tag{10.4}$$

A peptide with P_4-P_3-P_2-D motif is represented by a 20 × 3 matrix (c_{ij}) of 0 s and 1 s, where c_{ij}=1 if amino acid i is at position j.

Correlation analysis of the APF score and the binding energy in the analytical library were performed. If a high correlation is observed, it becomes possible to predict binding affinities from the APF score. The threshold value set up a value that makes the correlation coefficient between the APF score and the binding energy maximal. The predictive library was used to verify the predictability of the APF score.

10.4.1.4 Step 4: Construction of an Enriched Library by APF Score

It may be presumed that potent inhibitory peptides are distributed randomly in the comprehensive peptide library. The APF scores of all peptides in the library were calculated using APF score matrix. Since a high APF score indicates high binding affinity, peptides in the library were sorted in descending order according to their APF score. Thus, potent inhibitory peptides could be located at higher rank in the library. The enriched library was defined as the top 5% of the sorted comprehensive peptide library.

10.4.2 Design of Caspase-3-Specific Inhibitor by Using APF Method

We constructed the enriched library for caspase-3 inhibitors by using the APF method. Potent inhibitory peptides could be contained in the enriched library. Therefore, all peptides in the enriched library were docked into the active site of caspase-3 to calculate the binding energies. Ten predicted potent inhibitory peptides of caspase-3 are summarized in Table 10.2. The most potent inhibitory peptide was DEVD. DELD is the known cleavage site of D4-GDP dissociation

Table 10.2 Predicted inhibitory peptides against caspase-3

Ranking	Peptide	Binding energy (kcal/mol)	Substrate
1	DEVD	−16.15	PARP
2	QEPD	−15.91	–
3	TEPD	−15.75	–
4	DELD	−15.65	D4 GDP inhibitor
5	QELD	−15.63	–
6	LEPD	−15.57	–
7	DNLD	−15.45	–
8	DQPD	−15.39	–
9	DPFD	−15.16	–
10	NEPD	−14.95	–

Table 10.3 Potency and selectivity of caspase-3 inhibitors

Inhibitor	$K_i^{app\,a}$ (nM)			
	Caspase-3	Caspase-7	Caspase-8	Caspase-9
Ac-DNLD-CHO	0.68	55.7	>200	>200
Ac-DEVD-CHO	0.288	4.48	0.597	1.35
Ac-DQTD-CHO	1.27	21.8	9.75	14.5
Ac-DMQD-CHO	13.3	>200	>200	>200

[a]K_i^{app} was calculated from the IC_{50} value, $K_i^{app} = IC_{50}/(1+ [S]/K_m)$, where K_m is the Michaelis constant of the substrate and [S] is the substrate concentration in the assay

inhibitors [85]. Seven of the ten peptides had Glu in the P_3 position. Many of the well-known peptide inhibitors of caspases also have Glu in the P_3 position. Intriguingly, only DNLD had Asn in the P_3 position, and no peptide inhibitors or cleavage site in natural substrates of caspase-3 was known to have Asn in the P_3 position. Accordingly, we synthesized Ac-DNLD-CHO as a novel caspase-3 inhibitor. To determine inhibitory activities of Ac-DNLD-CHO against caspase-3, caspase-7, caspase-8, and caspase-9, in vitro caspase activity assays were performed (Table 10.3) [86]. Moreover, to evaluate the selectivity of Ac-DNLD-CHO against caspase-3, inhibitory activities of Ac-DEVD-CHO, Ac-DQTD-CHO, and Ac-DMQD-CHO were also examined. These three caspase inhibitors are commercially available. As shown in Table 10.3, Ac-DNLD-CHO inhibits caspase-3 (K_i^{app} = 0.68 nM) with almost the same potency as Ac-DEVD-CHO (K_i^{app} = 0.288 nM). Ac-DEVD-CHO inhibits caspase-3, caspase-7, caspase-8, and caspase-9 to similar extent. In contrast, Ac-DNLD-CHO has very low inhibitory activities against caspase-8 and caspase-9. Although Ac-DNLD-CHO inhibits caspase-7, Ac-DNLD-CHO exhibits about 80-fold selectivity for caspase-3 (K_i^{app} = 0.68 nM) over caspase-7 (K_i^{app} = 55.7 nM). Ac-DQTD-CHO did not exhibit great selectivity for caspase-3 over other caspases, and Ac-DMQT-CHO inhibits caspase-3 weakly (K_i^{app} = 13.3 nM), whereas it had little inhibitory effect on caspase-7, caspase-8, and caspase-9. The docking model of Ac-DNLD-CHO in complex with caspase-3 revealed characteristic interaction patterns with caspase-3 (Fig. 10.7a). The Asn (P_3) forms a hydrogen bond with Ser209 and does not interact with Arg207, while the Leu (P_2) forms tight hydrophobic interaction with Trp206, Tyr204, and Phe256 (S_2 site). Meanwhile, the Glu (P_3) in Ac-DEVD-CHO forms an interaction with the Arg207 but not Ser209 in the X-ray crystal structures of caspase-3 (PDB entry 1PAU). The hydrophobic interaction between the Val (P_2) in Ac-DEVD-CHO and the S_2 site is probably weaker than those between the Leu (P_2) in Ac-DNLD-CHO and the S_2 site. The specific inhibitory activity of Ac-DNLD-CHO against caspase-3 may arise from the characteristic interactions of the Asn (P_2) and the Leu (P_3). Non-peptidic small molecule, CS4566, selectively inhibits caspase-3, which is designed using pharmacophore derived from the specific binding mode of Ac-DNLD-CHO (Fig. 10.7b) [87]. Ac-DNLD-CHO and CS4566 represent promising lead structures for caspase-mediated diseases, such as neurodegenerative disorders.

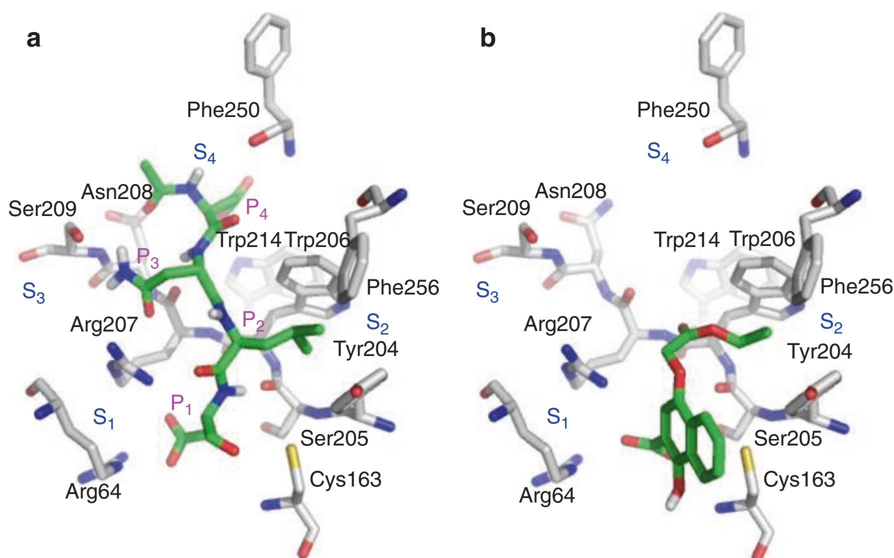


Fig. 10.7 Predicted binding interactions for Ac-DNLD-CHO (**a**) and CS4566 (**b**) on the active site of caspase-3. The active site of caspase-3 is represented in stick model with carbon colored *gray*, oxygen colored *red*, and nitrogen colored *blue*. Ac-DNLD-CHO and CS4566 are represented in stick model with carbon colored *green*, oxygen colored *red*, hydrogen colored *white*, and nitrogen colored *blue*

10.5 Conclusion

Since apoptosis-related proteins are attractive drug targets, many compounds that modulate these protein functions involved in apoptotic processes are currently being developed. In the apoptotic processes, protein-protein interactions (PPI) are very important in the regulation of the apoptotic signal transduction. However, targeting PPIs and creation of effective small molecules have long been considered to be difficult. Recently some successful examples are growing by peptide mimetic small molecules. For example, many of IAP antagonists were designed using the peptide sequence of IAP-binding motif. The IAP antagonists possess scaffolds that mimic a β-strand structure, while α-helix mimetics are also used for designing PPI inhibitors, such as Bcl-2 family antagonists and MDM2 inhibitors. Furthermore, NMR-based screening for identification of PPI inhibitors, such as Bcl-2 family antagonist ABT-737, is also an effective approach. Due to accumulation of crystal structures of PPI complexes, structure-based drug design strategies, such as our APF method, have become effective to create apoptosis-modulating PPI inhibitors. Thus, in recent years, significant advances have been made in the development of strategies to design PPI inhibitors. In the near future, apoptosis-modulating small molecules will be utilized in the chemotherapy of apoptosis-related diseases, such as cancers and neurodegenerative diseases.

References

1. Lockshin RA, Williams CM. Programmed cell death—I. Cytology of degeneration in the intersegmental muscles of the pernyi silkmoth. J Insect Physiol. 1965;11:123–33.
2. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer. 1972;26(4):239–57.
3. Häcker G. The morphology of apoptosis. Cell Tissue Res. 2000;301(1):5–17.
4. Toné S, Sugimoto K, Tanda K, et al. Three distinct stages of apoptotic nuclear condensation revealed by time-lapse imaging, biochemical and electron microscopy analysis of cell-free apoptosis. Exp Cell Res. 2007;313(16):3635–44.
5. Wyllie AH. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature. 1980;284(5756):555–6.
6. Kannan K, Jain SK. Oxidative stress and apoptosis. Pathophysiology. 2000;7(3):153–63.
7. Takasawa R, Nakamura H, Mori T, et al. Differential apoptotic pathways in human keratinocyte HaCaT cells exposed to UVB and UVC. Apoptosis. 2005;10(5):1121–30.
8. Roulston A, Marcellus RC, Branton PE. Viruses and apoptosis. Annu Rev Microbiol. 1999;53:577–628.
9. Fadeel B, Orrenius S, Zhivotovsky B. Apoptosis in human disease: a new skin for the old ceremony? Biochem Biophys Res Commun. 1999;266(3):699–717.
10. Kim TW, Pettingell WH, Jung YK, et al. Alternative cleavage of Alzheimer-associated presenilins during apoptosis by a caspase-3 family protease. Science. 1997;277(5324):373–6.
11. Guicciardi ME, Gores GJ. Apoptosis: a mechanism of acute and chronic liver injury. Gut. 2005;54(7):1024–33.
12. Barreyro FJ, Holod S, Finocchietto PV, et al. The pan-caspase inhibitor Emricasan (IDN-6556) decreases liver injury and fibrosis in a murine model of non-alcoholic steatohepatitis. Liver Int. 2015;35(3):953–66.
13. McIlwain DR, Berger T, Mak TW. Caspase functions in cell death and disease. Cold Spring Harb Perspect Biol. 2013;5(4):a008656.
14. Baskin-Bey ES, Washburn K, Feng S, et al. Clinical trial of the pan-caspase inhibitor, IDN-6556, in human liver preservation injury. Am J Transplant. 2007;7(1):218–25.
15. Plati J, Bucur O, Khosravi-Far R. Dysregulation of apoptotic signaling in cancer: molecular mechanisms and therapeutic opportunities. J Cell Biochem. 2008;104(4):1124–49.
16. Birchall MA, Winterford CM, Allan DJ, et al. Apoptosis in normal epithelium, premalignant and malignant lesions of the oropharynx and oral cavity: a preliminary study. Eur J Cancer B Oral Oncol. 1995;31B(6):380–3.
17. Weinstein RS, Manolagas SC. Apoptosis and osteoporosis. Am J Med. 2000;108(2):153–64.
18. Fulda S. Tumor resistance to apoptosis. Int J Cancer. 2009;124(3):511–5.
19. Hassan M, Watari H, AbuAlmaaty A, et al. Apoptosis and molecular targeting therapy in cancer. Biomed Res Int. 2014;2014:150845.
20. Deveraux QL, Reed JC. IAP family proteins—suppressors of apoptosis. Genes Dev. 1999;13(3):239–52.
21. Scott FL, Denault J-B, Riedl SJ, et al. XIAP inhibits caspase-3 and -7 using two binding sites: evolutionarily conserved mechanism of IAPs. EMBO J. 2005;24(3):645–55.
22. Shiozaki EN, Chai J, Rigotti DJ, et al. Mechanism of XIAP-mediated inhibition of caspase-9. Mol Cell. 2003;11(2):519–27.
23. Cai Q, Sun H, Peng Y, et al. A potent and orally active antagonist of multiple inhibitor of apoptosis proteins (IAPs) (SM-406/AT-406) in clinical development for cancer treatment. J Med Chem. 2011;54(8):2714–26.
24. Wu G, Chai J, Suber TL, et al. Structural basis of IAP recognition by Smac/DIABLO. Nature. 2000;408(6815):1008–12.
25. Kvansakul M, Hinds MG. Structural biology of the Bcl-2 family and its mimicry by viral proteins. Cell Death Dis. 2013;4:e909.

26. Kussie PH, Gorina S, Marechal V, et al. Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. Science. 1996;274(5289):948–53.
27. Wei Y, Fox T, Chambers SP, et al. The structures of caspases-1, −3, −7 and −8 reveal the basis for substrate and inhibitor selectivity. Chem Biol. 2000;7(6):423–32.
28. Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol. 2007;35(4):495–516.
29. Micheau O, Tschopp J. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell. 2003;114(2):181–90.
30. Waring P, Müllbacher A. Cell death induced by the Fas/Fas ligand pathway and its role in pathology. Immunol Cell Biol. 1999;77(4):312–7.
31. Schneider P, Thome M, Burns K, et al. TRAIL receptors 1 (DR4) and 2 (DR5) signal FADD-dependent apoptosis and activate NF-kappaB. Immunity. 1997;7(6):831–6.
32. Walczak H. Death receptor-ligand systems in cancer, cell death, and inflammation. Cold Spring Harb Perspect Biol. 2013;5(5):a008698.
33. Chang DW, Xing Z, Capacio VL, et al. Interdimer processing mechanism of procaspase-8 activation. EMBO J. 2003;22(16):4132–42.
34. Stennicke HR, Jürgensmeier JM, Shin H, et al. Pro-caspase-3 is a major physiologic target of caspase-8. J Biol Chem. 1998;273(42):27084–90.
35. Enari M, Sakahira H, Yokoyama H, et al. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature. 1998;391(6662):43–50.
36. Los M, Mozoluk M, Ferrari D, et al. Activation and caspase-mediated inhibition of PARP: a molecular switch between fibroblast necrosis and apoptosis in death receptor signaling. Mol Biol Cell. 2002;13(3):978–88.
37. Sahara S, Aoto M, Eguchi Y, et al. Acinus is a caspase-3-activated protein required for apoptotic chromatin condensation. Nature. 1999;401(6749):168–73.
38. Wolf BB, Schuler M, Echeverri F, et al. Caspase-3 is the primary activator of apoptotic DNA fragmentation via DNA fragmentation factor-45/inhibitor of caspase-activated DNase inactivation. J Biol Chem. 1999;274(43):30651–6.
39. Li H, Zhu H, CJ X, et al. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell. 1998;94(4):491–501.
40. Bellail AC, Qi L, Mulligan P, et al. TRAIL agonists on clinical trials for cancer therapy: the promises and the challenges. Rev Recent Clin Trials. 2009;4(1):34–41.
41. Wang G, Wang X, Yu H, et al. Small-molecule activation of the TRAIL receptor DR5 in human cancer cells. Nat Chem Biol. 2013;9(2):84–9.
42. Saelens X, Festjens N, Vande Walle L, et al. Toxic proteins released from mitochondria in cell death. Oncogene. 2004;23(16):2861–74.
43. Cain K, Bratton SB, Cohen GM. The Apaf-1 apoptosome: a large caspase-activating complex. Biochimie. 2002;84(2–3):203–14.
44. Tsujimoto Y. Role of Bcl-2 family proteins in apoptosis: apoptosomes or mitochondria? Genes Cells. 1998;3(11):697–707.
45. Shamas-Din A, Kale J, Leber B, et al. Mechanisms of action of Bcl-2 family proteins. Cold Spring Harb Perspect Biol. 2013;5(4):a008714.
46. Yip KW, Reed JC. Bcl-2 family proteins and cancer. Oncogene. 2008;27(50):6398–406.
47. Ding J, Zhang Z, Roberts GJ, et al. Bcl-2 and Bax interact via the BH1-3 groove-BH3 motif interface and a novel interface involving the BH4 motif. J Biol Chem. 2010;285(37):28749–63.
48. Lessene G, Czabotar PE, Colman PM. BCL-2 family antagonists for cancer therapy. Nat Rev Drug Discov. 2008;7(12):989–1000.
49. Liu Z, Sun C, Olejniczak ET, et al. Structural basis for binding of Smac/DIABLO to the XIAP BIR3 domain. Nature. 2000;408(6815):1004–8.
50. Gyrd-Hansen M, Meier P. IAPs: from caspase inhibitors to modulators of NF-kappaB, inflammation and cancer. Nat Rev Cancer. 2010;10(8):561–74.
51. Moll UM, Petrenko O. The MDM2-p53 interaction. Mol Cancer Res. 2003;1(14):1001–8.
52. Zhao Y, Aguilar A, Bernard D, et al. Small-molecule inhibitors of the MDM2-p53 protein-protein interaction (MDM2 inhibitors) in clinical trials for cancer treatment. J Med Chem. 2015;58(3):1038–52.

53. Shangary S, Johnson DE. Peptides derived from BH3 domains of Bcl-2 family members: a comparative analysis of inhibition of Bcl-2, Bcl-xL and Bax oligomerization, induction of cytochrome c release, and activation of cell death. Biochemistry. 2002;41(30):9485–95.
54. Petros AM, Nettesheim DG, Wang Y, et al. Rationale for Bcl-xL/Bad peptide complex formation from structure, mutagenesis, and biophysical studies. Protein Sci. 2000;9(12):2528–34.
55. Yin H, Lee GI, Sedey KA, et al. Terphenyl-based Bak BH3 alpha-helical proteomimetics as low-molecular-weight antagonists of Bcl-xL. J Am Chem Soc. 2005;127(29):10191–6.
56. Cao X, Yap JL, Newell-Rogers MK, et al. The novel BH3 α-helix mimetic JY-1-106 induces apoptosis in a subset of cancer cells (lung cancer, colon cancer and mesothelioma) by disrupting Bcl-xL and Mcl-1 protein-protein interactions with Bak. Mol Cancer. 2013;12(1):42.
57. Wang Z, Song T, Feng Y, et al. Bcl-2/MDM2 dual inhibitors based on universal pyramid-like α-helical mimetics. J Med Chem. 2016;59(7):3152–62.
58. Petros AM, Dinges J, Augeri DJ, et al. Discovery of a potent inhibitor of the antiapoptotic protein Bcl-xL from NMR and parallel synthesis. J Med Chem. 2006;49(2):656–63.
59. Oltersdorf T, Elmore SW, Shoemaker AR, et al. An inhibitor of Bcl-2 family proteins induces regression of solid tumours. Nature. 2005;435(7042):677–81.
60. Wei Y, Fan T, Yu M. Inhibitor of apoptosis proteins and apoptosis. Acta Biochim Biophys Sin Shanghai. 2008;40(4):278–88.
61. Shiraki K, Sugimoto K, Yamanaka Y, et al. Overexpression of X-linked inhibitor of apoptosis in human hepatocellular carcinoma. Int J Mol Med. 2003;12(5):705–8.
62. Sharma SK, Straub C, Zawel L. Development of peptidomimetics targeting IAPs. Int J Pept Res Ther. 2006;12(1):21–32.
63. Flygare JA, Beresini M, Budha N, et al. Discovery of a potent small-molecule antagonist of inhibitor of apoptosis (IAP) proteins and clinical candidate for the treatment of cancer (GDC-0152). J Med Chem. 2012;55(9):4101–13.
64. Brunckhorst MK, Lerner D, Wang S, et al. AT-406, an orally active antagonist of multiple inhibitor of apoptosis proteins, inhibits progression of human ovarian cancer. Cancer Biol Ther. 2012;13(9):804–11.
65. Varfolomeev E, Blankenship JW, Wayson SM, et al. IAP antagonists induce autoubiquitination of c-IAPs, NF-κB activation, and TNFα-dependent apoptosis. Cell. 2007;131(4):669–81.
66. Eschenburg G, Eggert A, Schramm A, et al. Smac mimetic LBW242 sensitizes XIAP-overexpressing neuroblastoma cells for TNF-α-independent apoptosis. Cancer Res. 2012;72(10):2645–56.
67. Benetatos CA, Mitsuuchi Y, Burns JM, et al. Birinapant (TL32711), a bivalent SMAC mimetic, targets TRAF2-associated cIAPs, abrogates TNF-induced NF-κB activation, and is active in patient-derived xenograft models. Mol Cancer Ther. 2014;13(4):867–79.
68. Seigal BA, Connors WH, Fraley A, et al. The discovery of macrocyclic XIAP antagonists from a DNA-programmed chemistry library, and their optimization to give lead compounds with in vivo antitumor activity. J Med Chem. 2015;58(6):2855–61.
69. Bieging KT, Mello SS, Attardi LD. Unravelling mechanisms of p53-mediated tumour suppression. Nat Rev Cancer. 2014;14(5):359–70.
70. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. Nature. 2000;408(6810):307–10.
71. Vassilev LT, BT V, Graves B, et al. In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. Science. 2004;303(5659):844–8.
72. Shangary S, Wang S. Small-molecule inhibitors of the MDM2-p53 protein-protein interaction to reactivate p53 function: a novel approach for cancer therapy. Annu Rev Pharmacol Toxicol. 2009;49:223–41.
73. Vu B, Wovkulich P, Pizzolato G, et al. Discovery of RG7112: a small-molecule MDM2 inhibitor in clinical development. ACS Med Chem Lett. 2013;4(5):466–9.
74. Ding Q, Zhang Z, Liu J-J, et al. Discovery of RG7388, a potent and selective p53–MDM2 inhibitor in clinical development. J Med Chem. 2013;56(14):5979–83.
75. Wade M, Wang YV, Wahl GM. The p53 orchestra: Mdm2 and Mdmx set the tone. Trends Cell Biol. 2010;20(5):299–309.

76. Shangary S, Qin D, McEachern D, et al. Temporal activation of p53 by a specific MDM2 inhibitor is selectively toxic to tumors and leads to complete tumor growth inhibition. Proc Natl Acad Sci U S A. 2008;105(10):3933–8.
77. Reed D, Shen Y, Shelat AA, et al. Identification and characterization of the first small molecule inhibitor of MDMX. J Biol Chem. 2010;285(14):10786–9.
78. Bista M, Smithson D, Pecak A, et al. On the mechanism of action of SJ-172550 in inhibiting the interaction of MDM4 and p53. PLoS One. 2012;7(6):e37518.
79. Graves B, Thompson T, Xia M, et al. Activation of the p53 pathway by small-molecule-induced MDM2 and MDMX dimerization. Proc Natl Acad Sci U S A. 2012;109(29):11788–93.
80. Rotonda J, Nicholson DW, Fazil KM, et al. The three-dimensional structure of apopain/CPP32, a key mediator of apoptosis. Nat Struct Biol. 1996;3(7):619–25.
81. Lazebnik YA, Kaufmann SH, Desnoyers S, et al. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. Nature. 1994;371(6495):346–7.
82. Garcia-Calvo M, Peterson EP, Leiting B, et al. Inhibition of human caspases by peptide-based and macromolecular inhibitors. J Biol Chem. 1998;273(49):32608–13.
83. Yoshimori A, Takasawa R, Tanuma S. A novel method for evaluation and screening of caspase inhibitory peptides by the amino acid positional fitness score. BMC Pharmacol. 2004;4:7.
84. Morris GM, Goodsell DS, Haliday RS, et al. Automated docking using a lamarckian genetic algorithm and an empirical binding free energy function. J Comp Chem. 1998;19(14):1639–62.
85. Na S, Chuang TH, Cunningham A, et al. D4-GDI, a substrate of CPP32, is proteolyzed during Fas-induced apoptosis. J Biol Chem. 1996;271(19):11209–13.
86. Yoshimori A, Sakai J, Sunaga S, et al. Structural and functional definition of the specificity of a novel caspase-3 inhibitor, Ac-DNLD-CHO. BMC Pharmacol. 2007;7:8.
87. Sakai J, Yoshimori A, Nose Y, et al. Structure-based discovery of a novel non-peptidic small molecular inhibitor of caspase-3. Bioorg Med Chem. 2008;16(9):4854–9.