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TGF-β in Human Disease

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Preface

More than 30 years after the discovery and purification of transforming growth factor-β (TGF-β) as a secreted polypeptide from oncogenically transformed cells, this multifunctional cytokine has led major research efforts in diverse fields ranging from embryonic development to adult organ physiology and pathobiology of major diseases, including cancer and fibrotic, cardiovascular, and immunological diseases. Over the years, intense research efforts have resulted in a deep understanding of the signaling pathway engaged by TGF-β and many of the regulatory mechanisms of its signaling engine that allow us today to explain specific aspects of human disease progression based on TGF-β. Despite such major achievements, the field of TGF-β signaling remains one of the most highly evolving and rapidly changing fields of modern biomedical science, a fact that is confirmed by the high rate and impact of the publications produced in the field. This activity underscores the current importance of exploiting modern technological tools to expand and deepen the investigation of TGF-β pathway perturbations in human disease. This ultimate goal of the scientific field is feasible but far from being achieved.

The present book aims to provide a comprehensive coverage of major areas of human disease where the involvement of TGF-β and the mechanistic detail of understanding are firmly established. Simultaneously, the book aims to highlight major gaps of knowledge and future directions of research that can benefit human medical science. A good example is the strong potential of translational application of the TGF-β pathway in therapy for diverse human disorders. In reaching our goals, the book encompasses a broad series of topics with reference to specific human diseases. Owing to the vast number of human disorders where TGF-β is implicated, not all diseases are listed in the contents of this book. Furthermore, as TGF-β is a member of a large family of 33 cytokines that exhibit similarities but also unique features, the book often makes reference to some of the other growth factors of the TGF-β family but it does not comprehensively cover all of them. The core set of diseases where TGF-β action is well documented and are included in the book are cancer and cardiovascular and fibrotic disorders.

Our ambition is that this book will stimulate young scientists to enter the prolific TGF-β field and find new solutions to the many open problems remaining in this

area of study. For this reason we have made a special effort to include authoritative educational chapters that provide a good introduction to the field for young doctoral students, postdocs, and clinical fellows. We believe that the book will also be a valuable reference for the pioneers and aficionados of the field, who can find accessible and well-illustrated material for their teaching, lecturing, and other activities, via which the importance of TGF-β biology needs to be disseminated to the world of science and to the public. To meet all these goals, we as editors recruited a select group of world leaders in specific subjects who have delivered outstanding examples of scientific literature. We are in debt to the meticulous work of all these authors and thank them for helping us realize our goal.

Uppsala, Sweden — Aristidis Moustakas
Yamanashi, Japan — Keiji Miyazawa

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Part I
Introduction

Chapter 1
Transforming Growth Factor-β Signaling

Carl-Henrik Heldin

Abstract Members of the transforming growth factor β (TGF-β) family regulate cell proliferation, migration, and differentiation during embryonal development and in tissue homeostasis in the adult. They signal by inducing heteromeric complexes of type I and type II serine/threonine kinase receptors. Ligand binding activates the type I receptor kinase leading to phosphorylation of members of the Smad family, which after oligomerization are translocated to the nucleus where they together with other nuclear factors regulate the transcription of specific genes. TGF-β family members also signal via non-Smad pathways, including Erk, JNK, and p38 MAP-kinase pathways, the tyrosine kinase Src, the small GTPase Rho, and cleavage of the type I receptor whereby the intracellular domain is translocated to the nucleus where it drives an invasiveness program. The TGF-β signaling pathways are carefully regulated by posttranslational mechanisms, including phosphorylation, ubiquitination, acetylation, sumoylation, and PAR-ylation, as well as by positive and negative feedback mechanisms and cross talk with other signaling pathways.

Keywords Posttranslational modifications • Serine/Threonine kinase receptors • Signal transduction • Smad molecules • TGF-β

1.1 Introduction

The transforming growth factor-β (TGF-β) family of ligands has 33 members in humans, including TGF-β isoforms, activins, nodal, bone morphogenetic proteins (BMPs), and growth and differentiation factors (GDFs) (Derynck and Miyazono 2007). They have important roles as morphogens during embryonal development

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and in the regulation of tissue homeostasis in the adult (Moustakas and Heldin 2009; Wu and Hill 2009). TGF-β family members are pluripotent and regulate cell growth, migration, death, and differentiation; aberrant signaling has been linked with various diseases, such as autoimmune diseases, cardiovascular diseases, and cancer. This review will focus on the mechanisms whereby TGF-β isoforms signal via Smad and non-Smad pathways. A remarkable aspect of TGF-β signaling, which will be discussed, is that the magnitude and duration of signaling is carefully controlled at many different levels, including the synthesis and activation of latent TGF-β isoforms, receptor activation and stability, and the activation and stability of Smad molecules and other downstream signaling molecules.

1.2 TGF-β Ligands

The three TGF-β isoforms are synthesized as precursor molecules that are secreted in latent forms and need to be activated before they can act on their target cells. The about 400 amino acid residue long precursors dimerize and are cleaved by furin-like proteases during secretion; the C-terminal TGF-β molecule thereafter remains bound to the N-terminal part of the precursor, the latency associated peptide (LAP). Within the LAP molecule, an α helix and a "latency lasso" trap TGF-β like a "straitjacket" (Shi et al. 2011). The latent TGF-β complex often forms larger complexes with certain members of the latent TGF-β binding protein (LTBP) family of fibrillin-like molecules that confer interactions with components of the extracellular matrix (Hyytiäinen et al. 2004). TGF-β isoforms can be released from the latent complexes by exposure to low or high pH, or, more physiologically, by cleavage of LAP by certain proteases, by competition by certain matrix molecules, or by physical forces (Annes et al. 2003). Integrins have been shown to have a central role in TGF-β activation (Nishimura 2009); on the one hand integrins guide proteases to the latent complex, and on the other hand they transmit traction forces which lead to release of TGF-β from the latent complex (Buscemi et al. 2011).

The synthesis of TGF-β isoforms are controlled by external stimuli. Moreover, sortilin, which is structurally related to the yeast vacuolar protein sorting 10 (Vps10p) negatively regulates TGF-β signaling by diverting trafficking of precursor proteins to the lysosomes during transit through the biosynthetic pathway (Kwon and Christian 2011).

1.3 Signaling via TGF-β Receptors

TGF-β isoforms exert their effects on cells by binding to heterotetrameric complexes of two type I and two type II serine/threonine kinase receptors (Fig. 1.1). Altogether there are seven type I and five type II serine/threonine kinase receptors for TGF-β family ligands in humans. All three TGF-β isoforms bind to the TGF-β

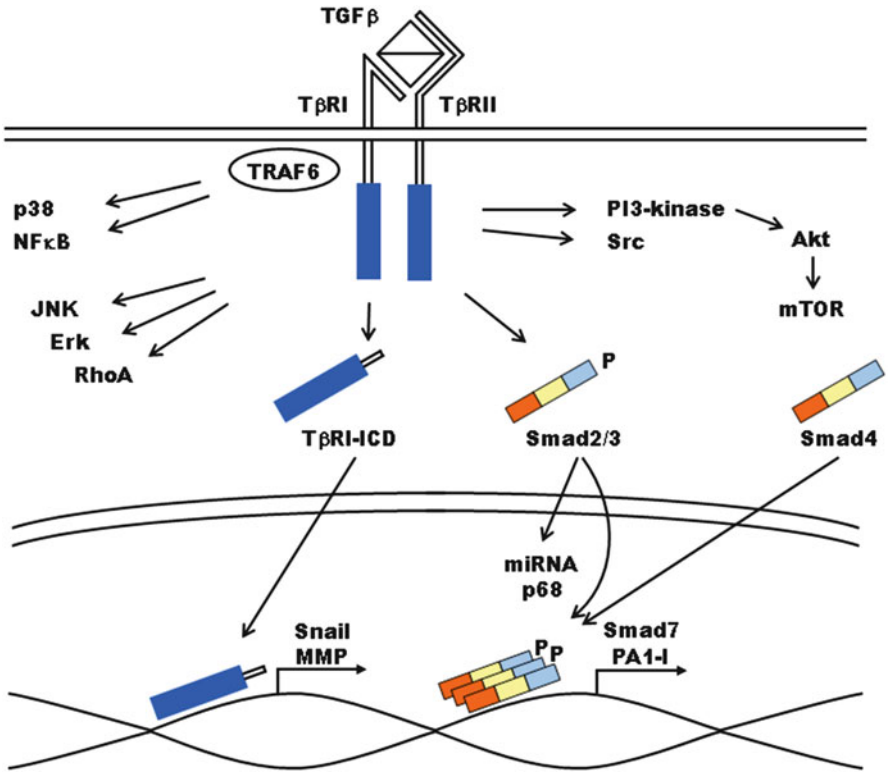

Fig. 1.1 Schematic illustration of major signaling pathways downstream of TGF-β receptors. For explanation, see the text

type II receptor (TβRII) and the TGF-β type I receptor (TβRI; ALK5) that are present on most cell types; in addition, they bind to another type I receptor (ALK1) that are preferentially present on endothelial cells (Goumans et al. 2003).

After TGF-β has induced a complex of TβRII and TβRI, TβRII phosphorylates TβRI in the GS domain located just upstream of the kinase domain (Wrana et al. 1994). Thereby an auto-inhibitory mechanism is perturbed and the TβRI kinase is activated and ready to phosphorylate its substrates, including members of the Smad family. In the ligand-receptor complex, TGF-β signaling is mediated by two autonomously functioning TβRI:TβRII pairs (Huang et al. 2011). Receptor activation by phosphorylation is counteracted by dephosphorylation by the PP2A phosphatase. This phosphatase contains a B regulatory subunit which occurs as two isoforms; the Bα subunit promotes and the Bδ subunit suppresses TGF-β signaling (Batut et al. 2008). Signaling via TβRI is moreover enhanced by sumoylation of the receptor, which possibly promotes the docking and phosphorylation of Smad molecules (Kang et al. 2008).

1.4 TGF-β Co-receptors

The interaction of TGF-β with TβRI and TβRII is enhanced by certain co-receptors, such as the proteoglycan TβRIII (also called betaglycan) and endoglin which is expressed preferentially on endothelial cells (Pardali et al. 2010). TβRIII undergoes ectodomain shedding whereby the extracellular domain is released by proteolysis and then act as an antagonist by scaffolding TGF-β and preventing it from binding to TβRI and TβRII (López-Casillas et al. 1994).

In keratinocytes, TGF-β signaling is negatively modulated by the glycosylphosphatidylinositol-anchored protein CD109, which is a member of the α2-macroglobulin family (Tam et al. 2003). The mechanism whereby CD109 exerts its negative effect on TGF-β signaling may involve promotion of TGF-β receptor localization in lipid rafts of the cell membrane and promotion of receptor degradation (Bizet et al. 2012).

The tetraspanin protein CD151/Tspan24, which interacts with integrins and many other receptor types, enhances TGF-β signaling; CD151 appears not to bind TβRI directly but may indirectly affect TβRI distribution (Sadej et al. 2010).

1.5 Functional Domains of Smad Molecules

Members of the Smad family of signal transducers are important substrates for serine/threonine kinase receptors (Fig. 1.2). They have conserved Mad homology (MH)1 and MH2 domains connected by a linker region. The N-terminal MH1 domain has a β-hairpin loop which can bind to DNA, and the C-terminal MH2 domain mediates interaction with receptors, other Smad isoforms and many other molecules (Moustakas and Heldin 2009). The linker region is subject to posttranslational modifications which affect interactions and the stability of Smad molecules.

The receptor-activated (R-)Smads are phosphorylated by the type I receptors in SXS motifs in their extreme C-terminals. The conventional TβRI and the type I activin receptor phosphorylate Smad2 and 3, whereas Smad1, 5, and 8 are phosphorylated by the receptors for most of the type I receptors for BMP and GDF isoforms, as well as ALK1. The activated R-Smads then form complexes with the common-mediator (Co-)Smad (Smad4), usually consisting of two R-Smad molecules and one Smad4 molecule; they are then translocated to the nucleus where they in cooperation with other nuclear factors regulate the transcription of certain genes (see below). Smad2 and Smad3 are 92 % identical in their sequences, but Smad2 has two extra sequences inserted in the MH1 domain which perturb DNA binding, thus giving the two molecules different functional effects.

Members of the subfamily of inhibitory (I-)Smads (Smad6 and 7) have the MH2 domain conserved but do not have any MH1 domain. They are induced after activation of serine/threonine kinase receptors and take part in a negative feedback

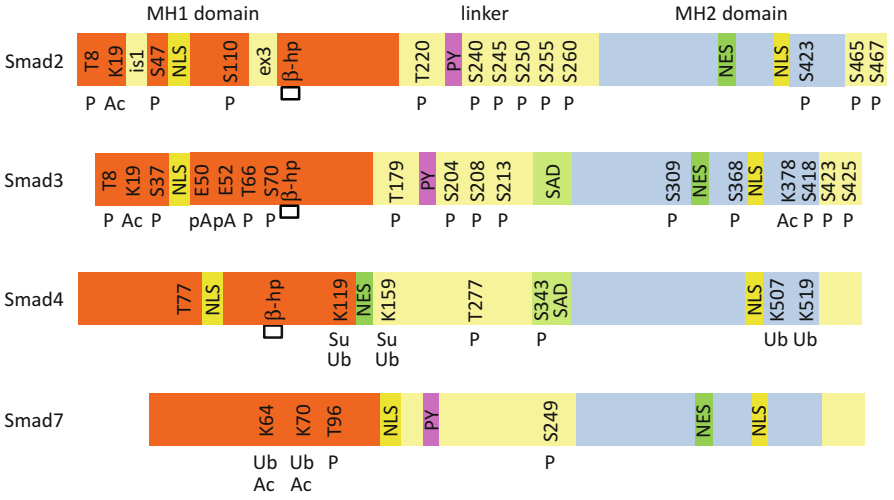

Fig. 1.2 Schematic illustration of the domain structures of the Smad molecules that are involved in TGF-β signaling. Amino acid residues undergoing posttranslationational modifications are indicated. *Is1* inserted sequence 1, *ex3* exon3, *NLS* nuclear localization sequence, *NES* nuclear export sequence, *PY* proline-tyrosine motif, *SAD* Smad activation domain, *β-hp* β-hairpin loop, *P* phosphorylation, *Ac* acetylation, *Ub* ubiquitination, *Su* sumoylation, *pA* poly-ADP-ribosylation

mechanism (see below). Whereas Smad7 interacts with both TGF-β receptors and BMP receptors, Smad6 is selective for BMP receptors.

1.6 Internalization of TGF-β Receptors

TGF-β receptor activation is followed by internalization via clathrin-coated pits into endosomes where the TGF-β receptor complex meets the R-Smad molecules which are delivered to them bound to SARA, a FYVE domain protein that resides in endosomes (Tsukazaki et al. 1998). Trafficking of TGF-β receptors to the endosomal compartment thus enables Smad activation (Hayes et al. 2002; Penheiter et al. 2002). The leucine-rich-repeat- and PDZ domain-containing protein ERBIN can compete with SARA for binding of Smad2 and 3 and thus modulates Smad activation (Sflomos et al. 2011).

Most of the endocytosed TGF-β receptor complexes are recycled back to the cell membrane to serve again in a process that is carefully regulated by RIN1, a Rab5 GTP-exchange factor (GEF) (Hu et al. 2008). This continuous endocytosis and recycling depletes ligand availability and is an important mechanism to fine-tune signaling (Clarke et al. 2009).

In addition to the clathrin-mediated internalization, TGF-β receptors can also be internalized via caveolae which leads to degradation of the receptor complex

(Di Guglielmo et al. 2003). Receptors entering this pathway interact with Smad7 carrying the ubiquitin ligases Smurf1 and 2, which through poly-ubiquitination mark the receptors for proteasomal degradation (see below). This process is controlled by the chaperone protein HSP90 which by binding to the receptors can prevent them from ubiquitination and degradation (Wrighton et al. 2008). Moreover, the calcium/phospholipid-binding protein Annexin A1 positively regulates TGF-β signaling possibly by interfering with TGF-β receptor endocytosis (de Graauw et al. 2010).

1.7 Control of TGF-β Receptor Signaling

TGF-β signaling induces Smad7 which exerts a negative feedback control on TGF-β signaling by competing with R-Smads for interaction with the receptors thereby lowering R-Smad phosphorylation (Hayashi et al. 1997; Kamiya et al. 2010; Nakao et al. 1997), by binding ubiquitin ligases of the Smurf family and bringing them to the receptors which thereby become polyubiquitinated and marked for proteasomal degradation (Kavsak et al. 2000; Zhu et al. 1999), and by binding the PP1C phosphatase and bringing it to the receptors thereby de-phosphorylating and de-activating the receptors (Shi et al. 2004). The negative feedback effect of Smad7 is balanced by TGF-β-induction of TGF-β-stimulated clone 22 (TSC22), which competes with Smad7 for binding to TβRI and thus promotes TGF-β signaling in a positive feedback mechanism (Yan et al. 2011).

The AMP-regulated kinase (AMPK) family member salt-inducible kinase (SIK), which is induced in parallel to Smad7 after TGF-β stimulation, binds to Smad7 and promotes receptor ubiquitination and degradation in cooperation with the ubiquitin ligase Smurf2 (Kowanetz et al. 2008; Lönn et al. 2012).

The ubiquitinated TGF-β receptors can be de-ubiquitinated by the de-ubiquitinase USP4 which binds directly to TβRI; the serine/threonine kinase Akt1 which is activated in a phosphatidylinositol 3′-kinase (PI3-kinase)-dependent manner by TGF-β and other growth factors, phosphorylates USP4 whereby it is re-located to the plasma membrane and promotes TβRI stability and TGF-β signaling (Zhang et al. 2012b). USP15, another de-ubiquitinase which interacts with the Smad7-Smurf2 complex, can also de-ubiquitinate TβRI and thus promote TGF-β signaling (Eichhorn et al. 2012). Interestingly, the USP15 gene has been found to be amplified in glioblastoma and the level of expression correlates with poor prognosis.

Smurf 1 and 2 bind to the PY motifs of I-Smads (and to R-Smads; see below); in addition to promoting ubiquitination and degradation of the TGF-β receptors, they promote ubiquitination and destabilization of Smad7 itself. In addition, the E3 ligases RNF12 (Zhang et al. 2012a) and Arkadia (Koinuma et al. 2003) bind to Smad7 and mediate its ubiquitination and destabilization; since these ubiquitin ligases do not ubiquitinate the receptors, they promote TGF-β signaling by weakening the negative feedback effect of Smad7. The E3 ligase Itch/AIP4 also binds to Smad7 and ubiquitinates it; however, it inhibits TGF-β signaling presumably by enhancing the interaction between Smad7 and TβRI (Lallemand et al. 2005).

1.8 Control of Smad Signaling by Posttranslational Modifications

The activity and stability of Smad proteins are carefully controlled by different mechanisms, including posttranslational modifications, e.g., phosphorylation, ubiquitination, sumoylation, acetylation, and poly-ADP-ribosylation (PAR-ylation) (Fig. 1.2).

1.8.1 Phosphorylation

In addition to the activating phosphorylation by type I receptors in the C-terminals, R-Smads have been shown to be C-terminally phosphorylated after hepatocyte growth factor stimulation, although the kinase involved has not been identified (de Caestecker et al. 1998). Moreover, Mps1, a kinase of the spindle checkpoint, has been shown to bind to Smad4, and activation of Smad2 in response to disruptions of the microtubule network by nocodazole (Dong et al. 2000) has been shown to be dependent on Mps1 (Zhu et al. 2007). Members of the WNK family of kinases have also been shown to phosphorylate R-Smads in their C-terminals, but they also phosphorylate other sites in Smads and thus have both positive and negative effects of the activity of Smads (Lee et al. 2007a).

R-Smads are also subject to negative regulatory phosphorylations; the Erk MAP-kinase phosphorylates them in the linker region, which prevents their accumulation in the nucleus (Kretzschmar et al. 1999). Other kinases that phosphorylate Smad2 or Smad3 in their linkers and suppress their activity include cyclin-dependent kinases (CDKs; Matsuura et al. 2004), p38 MAP-kinase (Kamaraju and Roberts 2005), JNK MAP-kinase (Mori et al. 2004), TGF-β-activated kinase 1 (TAK-1; Benus et al. 2005), MKP38 (Seong et al. 2010), and G-protein-coupled receptor kinase-2 (GRK-2; Ho et al. 2005).

Inhibitory phosphorylations occur also in the MH1 domain. Thus, phosphorylation of the MH1 domains of Smad2 or Smad3 by protein kinase C (PKC) interferes with DNA binding of Smad3 and thus inhibits Smad signaling (Yakymovych et al. 2001). Interestingly, the analogous amino acid residue in Smad4 can be phosphorylated by LKB1, which suppresses Smad4 activity (Morén et al. 2011). The calcium/calmodulin-dependent protein kinase II (CAMKII) phosphorylates Smad2 and Smad3, both in the MH1 domains and in the linkers, which inhibits Smad signaling (Wicks et al. 2000). In addition, glycogen synthase kinase 3β (GSK3β) phosphorylates the MH1 domain of Smad3, but not Smad2, which promotes its ubiquitination and proteasomal degradation (Guo et al. 2008). On the other hand, the Drosophila kinase Misshapen and its mammalian homologs, TNIK, MINK1, and MAPK4, phosphorylate all R-Smads, except Smad3, in their α-helix1 region and inhibit Smad signaling (Kaneko et al. 2011).

The activity of Smad4 is affected by phosphorylation by Erk MAP-kinase in a region in the linker referred to as the Smad activation domain which binds co-activators such as p300 and CBP; this phosphorylation enhances Smad signaling (Roelen et al. 2003). Interestingly, Smad7 can be phosphorylated in the corresponding region, whereas this phosphorylation does not affect the inhibitory effect of Smad7, it does influence its effect on transcription (Pulaski et al. 2001).

R-Smads are de-activated by de-phosphorylation of their C-terminal phosphorylations. Certain phosphatases dephosphorylate Smads in the nucleus, including PPM1A (also called PP2Cα) (Duan et al. 2006; Lin et al. 2006), which thus control the termination of Smad signaling. During hypoxia, PP2A selectively dephosphorylates Smad3 but not Smad2 (Heikkinen et al. 2010b). Other phosphatases, including the FYVE domain containing MTMR4 (Yu et al. 2010), reside in endosomes and thus titrate the magnitude of Smad activation. The inhibitory linker phosphorylation sites are dephosphorylated by small C-terminal domain phosphatases (SCP) 1, 2, and 3; these phosphates do not dephosphorylate the activating C-terminal phosphorylations (Sapkota et al. 2006; Wrighton et al. 2006).

1.8.2 Ubiquitination

The stability of R-Smads is affected by polyubiquitination by different E3 ligases. Some of these ubiquitin ligases act on non-activated Smads and thus titrate the steady state level of R-Smads, whereas others recognize activated Smads and thus contribute to termination of signaling.

Among the E3 ligases that act on nonactivated Smads are the HECT ubiquitin ligases Smurf1 and Smurf2 that bind to PY motifs in the linker regions of Smad1/5 and Smad1/2/3, respectively (Lin et al. 2000; Zhang et al. 2001; Zhu et al. 1999), Nedd4-2/Nedd4L (Kuratomi et al. 2005), Tiul/WWP1 (Komuro et al. 2004; Seo et al. 2004), WWP2 (Soond and Chantry 2011), SCF-ROC1 (Fukuchi et al. 2001) and CHIP (Xin et al. 2005).

Ubiquitin-mediated degradation of R-Smads is likely to be of major importance for termination of TGF-β signaling. TGF-β stimulation induces phosphorylations in the nucleus of the linker regions of Smad2 and Smad3 by CDK8 and CDK9 which serve as priming events for phosphorylation by GSK3 (Alarcón et al. 2009; Millet et al. 2009). The phosphorylations first enhance Smad transcriptional activity, then trigger ubiquitination of Smads by Nedd4-2/Nedd4L followed by proteasomal degradation (Aragon et al. 2011; Gao et al. 2009).

Recent studies have revealed that Smurf2 causes multiple mono-ubiquitination of Smad3 which prevents the formation of Smad complexes (Tang et al. 2011). Thus, ubiquitination can limit Smad signaling both by promoting Smad degradation and by inhibiting Smad interactions. The ubiquitinated R-Smads can be de-ubiquitinated by the de-ubiquitinase USP15 (Inui et al. 2011)

Smad4 is also subject to ubiquitination. The SCFβ^{TrCP} E3 ligase (Wan et al. 2004) and the E3 ligase JAB1/CSN5 (Wan et al. 2002) promote degradation of wild-type Smad4, whereas another member of the SCF family, SCF^{Skp2}, does not affect wild-type Smad4, but promotes degradation of mutated Smad4 found in cancers (Liang et al. 2004). Another ubiquitin ligase, ectodermin/TRIMM33/TIF1γ of the RING family, has been shown to be an E3 ligase for Smad4 (Dupont et al. 2005). Ubiquitination of Smad4 not only controls its stability but also affects its activity. Thus, mono-ubiquitination of Lys507 in Smad4 enhances its activity (Morén et al. 2003). Smad4 mono-ubiquitination can also occur on Lys519 by ectodermin/TRIMM33/TIF1γ which causes export of Smad4 from the nucleus and inhibition of signaling (Dupont et al. 2009). TGF1γ is also involved in epigenetic regulation of Smad signaling (see below). The ubiquitination of Smad4 is removed by the de-ubiquitinase FAM/USP9X (Dupont et al. 2009); other de-ubiquitinases have also been shown to act on ubiquitinated Smads, including UCH37/UCHL5 (Wicks et al. 2005).

1.8.3 Sumoylation

Smad4 has been shown to be sumoylated on Lys113 and Lys159 by the E3 ligase PIAS1. Sumoylation of these residues protects them from ubiquitination and thus prevents proteasomal degradation, thereby enhancing Smad4 signaling (Lee et al. 2003; Lin et al. 2003; Ohshima and Shimotohno 2003). However, there are also observations suggesting that sumoylation suppresses the effect of Smad4; thus, the adaptor protein Daxx represses Smad4 function by binding to sumoylated Lys159 (Chang et al. 2005). It is possible that the mechanism involves Daxx-dependent recruitment of histone deacetylases or silencing factors. Sumoylation has also been shown to promote nuclear export of Smad3, thus repressing signaling (Imoto et al. 2008).

1.8.4 Acetylation

The same lysine residues in Smad7 that can be ubiquitinated, i.e. Lys64 and Lys70, can alternatively be acetylated by the co-activator p300 (Grönroos et al. 2002). Thus, acetylation prevents ubiquitination and stabilizes Smad7. Thus, both sumoylation and acetylation can compete with ubiquitination and thereby fine-tune signaling. The acetyl groups on Smad7 can be removed by de-acetylases (Simonsson et al. 2005). Among the R-Smads, Smad2, but not Smad3, has been shown to be acetylated by p300 in the MH1 domain, in a manner that promotes TGF-β signaling (Simonsson et al. 2006; Tu and Luo 2007).

1.8.5 PAR-ylation

TGF-β stimulation promotes an interaction of Smad3 and Smad4 with poly-ADP-ribose-polymerase 1 (PARP1), whereby they are PAR-ylated. This decreases the binding of Smads to DNA and thus contributes to termination of signaling (Lönn et al. 2010).

1.9 Positive Control of Smad Signaling

In addition to C-terminal phosphorylation by TβRI, Smad signaling is enhanced by other mechanisms. After phosphorylation of Thr179 in the linker of Smad3, it binds the peptidyl-prolyl *cis*/*trans* isomerase PIN1. Whereas the knockdown of PIN1 had no effect on TGF-β-induced growth inhibition, it inhibited N-Cadherin expression, as well as migration and invasion of PC3 prostate cancer cells (Matsuura et al. 2010). In contrast, a suppressive role of PIN1 has also been observed; PIN1 was found to enhance the binding of Smurf2 to Smad2/3, resulting in enhanced ubiquitination and degradation (Nakano et al. 2009).

Smad3 also binds the pseudokinase Tribbles homolog 3 (TRB3) with its MH2 domain, thereby promoting nuclear localization of Smad3, possibly by preventing Smad3 from interacting with exportin 4 (Hua et al. 2011). Moreover, TBR3 binds to Smurf2 and promotes its degradation, thus enhancing Smad signaling further by limiting the ubiquitination and degradation of Smads.

1.10 Negative Control of Smad Signaling

In addition to posttranslational modifications, several other mechanisms control the magnitude and duration of Smad signaling. Thus, the transmembrane prostate androgen-induced protein (TMEPAI) interacts with R-Smads and prevents their binding to SARA, thereby suppressing Smad activation (Watanabe et al. 2010).

Smad signaling is also affected by mechanisms that change the amount of Smad molecules. For instance, the level of Smad3 is modulated by Ras activation and the levels of Smad3 are dramatically reduced in tumor cell lines with activated H-Ras (Daly et al. 2010). The mechanism involves effects on the mRNA level as well as stability of Smad3.

The zebrafish Piwi protein Zili suppresses Smad signaling by binding Smad4 and preventing complex formation with R-Smads (Sun et al. 2010); this control mechanism is important during early embryogenesis.

SnoN and Ski are negative regulators of TGF-β signaling which repress the transcriptional activities of Smad complexes by recruiting co-repressor complexes and blocking the interaction between Smads and co-activators (Akiyoshi et al. 1999; Luo et al. 1999; Stroschein et al. 1999; Wu et al. 2002). Moreover, Ski binds to TβRI and suppresses Smad activation (Ferrand et al. 2010).

1.11 Nucleocytoplasmic Shuttling of Smads

In order to perform their tasks as transcription factors, Smads need to be translocated to the nucleus. The nucleocytoplasmic shuttling of Smads is carefully controlled by different mechanisms for the various Smads molecules. Activation of R-Smads by receptor-mediated phosphorylation promotes nuclear accumulation, and it is likely that the time the Smad complexes spend in the nucleus determines the strength of the signaling (Inman et al. 2002; Nicolas et al. 2004; Schmierer and Hill 2005; Schmierer et al. 2008).

Among the TGF-β R-Smads, Smad3 has a putative nuclear localization signal (NLS) in the MH1 domain. Phosphorylated Smad3 interacts with importin-β1 of the nuclear pores and is taken into the nucleus by a mechanism that is dependent on the small GTPase Ran (Kurisaki et al. 2001; Xiao et al. 2000a; Xiao et al. 2000b). However, the NLS of Smad2 cannot interact with importin-β1 because of the inserted sequences in its MH1 domain (Kurisaki et al. 2001). Instead nuclear translocation is promoted by an epitope in the MH2 domain which can mediate interactions with FG-repeat-containing nucleoporins, such as CAN/Nup214 (Xu et al. 2000; Xu et al. 2002). Importin 7 and importin 8 have also been implicated in the nuclear import of Smad2, 3, and 4 (Xu et al. 2007; Yao et al. 2008). Moreover, Smad2 has been shown to be transported by kinesin-1 motors along microtubules through the cytoplasm; this transport mechanism is essential for Smad2 nuclear signaling (Batut et al. 2007). The export of Smad3 is dependent on a nuclear export sequence in the MH2 domain and on the Ran GTPase and exportin 4 (Kurisaki et al. 2006) or Ran binding protein 3 (RANBP3) (Dai et al. 2009); the mechanism of nuclear export of Smad2 is not known.

Several mechanisms have been proposed for the translocation of Smad4 into the nucleus. Thus, Smad4 can enter the nucleus in complex with R-Smads, by use of an NLS in the MH1 domain that interacts with importin-α (Pierreux et al. 2000; Xiao et al. 2003), or by interaction with CAN/Nup214 (Xu et al. 2003). The export of Smad4 is dependent on a nuclear export sequence in its linker region and involves binding to the exportin CRM1 (Pierreux et al. 2000; Watanabe et al. 2000).

There are also other mechanisms that regulate the nuclear residence of Smad complexes. Thus, the transcriptional co-activator with PDZ motif (TAZ) binds Smad complexes and anchors them at the chromatin by interaction with the anchor-recruited co-factor (ARC) protein ARC105 (Varelas et al. 2008).

In non-stimulated cells, Smad7, which has an NLS in its N-terminus, resides mainly in the nucleus. Upon TGF-β stimulation, Smad7 is exported from the nucleus (Itoh et al. 1998) in complex with Smurf1 (Ebisawa et al. 2001) or Smurf2 (Kavsak et al. 2000), which have NES epitopes and interact with CRM1 (Tajima et al. 2003).

1.12 MicroRNAs in Smad Signaling

Recent findings have shown that miRNAs have important roles in TGF-β signaling. Firstly, Smads have been shown to bind to the RNA helicase p68, which is a component of the Drosha complex that processes precursor miRNAs (Davis et al. 2008).

Moreover, Smads bind to SBE sequences in the stem structures of pri-miRNAs and thereby facilitates Drosha-mediated maturation of miRNAs (Davis et al. 2010).

Secondly, TGF-β induces several different miRNAs, including miR143/145 promoting smooth muscle cell differentiation via targeting of the transcription factor Klf4 (Davis-Dusenbery et al. 2011; Long and Miano 2011), miR-491-5p which targets the tight junction protein PAR-3 in proximal tubular epithelial cells thus disrupting cell junctions (Zhou et al. 2010), and miR-200 family members which regulate ZEB1 and ZEB2 that in turn have important roles in the regulation of EMT (Ahn et al. 2012).

Finally, Smad signaling is modulated by certain miRNAs. Thus, miR-155 targets Smad2 (Louafi et al. 2010) and miR-130a targets Smad4 (Häger et al. 2011), leading to decreased levels of these proteins and attenuated signaling.

1.13 Smads as Transcription Factors

Microarray analyses have revealed that TGF-β stimulation affects the transcription of several hundreds of genes. R-Smad/Smad4 complexes are of major importance as indicated by the dramatic effect of Smad4 knockdown on the transcriptional effects of TGF-β (Kowanetz et al. 2004).

1.13.1 DNA Binding of Smads

Smad3 and 4 bind DNA by an 11-amino-acid β-hairpin in their MH1 domains which contacts the major groove of DNA at the half-site 5′-GTCT-3′ and its reverse 5′-AGAC-3′ (Shi et al. 1998; Zawel et al. 1998). Whereas the most common form of Smad2 cannot bind DNA because of an inserted sequence immediately adjacent to the β-hairpin (Dennler et al. 1998; Shi et al. 1998), there is a splice variant of Smad2, lacking the inserted sequence, Smad2Δexon3, that does bind DNA (Yagi et al. 1999).

Since the DNA binding motif is short and thus common in the genome, and since DNA binding of Smads occurs at low affinity, the Smads are dependent on interactions with other transcription factors for their specificity.

1.13.2 Cooperation of Smads with Other Nuclear Factors

After Smad complexes and their transcriptional partners have bound to DNA, co-activators, such as the histone acetyltransferases p300 and P/CAF, are recruited, which facilitates initiation of transcription. Recent findings suggest that Smads bound to chromatin needs chromatin remodeling factors, such as Brahma-related gene 1 (BRG1) and ARC105 (Schmierer and Hill 2007; Xi et al. 2008).

Even if the most common configuration of Smad complexes is two R-Smad molecules and one Smad4 molecule, there are reports that the Smad4 molecule in the complexes may be replaced by ectodermin/TRIMM33/TGF1γ in the regulation of hematopoietic differentiation (He et al. 2006). In TIF1γ-depleted cells, Smad4 is more available for association with Smad2/3, leading to an enhanced TGF-β signaling which promotes EMT (Hesling et al. 2011). Moreover, in the epidermis of Smad4-null mice, the IκB kinase α (IKKα), which regulates the nuclear factor κB (NFκB) pathway, can form complexes with Smad2 and 3 and regulate keratinocyte differentiation by binding to the promoters of *Mad1* and *Ovol1* (Descargues et al. 2008).

By interacting with specific transcriptional co-factors, Smad complexes can induce groups of genes that in a coordinated manner regulate a specific response. Examples of such synexpression are the FoxO transcription factors that together with Smads regulate 11 genes that define the cytostatic, apoptotic, and adaptive response of keratinocytes (Gomis et al. 2006), the helix-loop-helix protein human homolog of Maid (HHM) which regulates a synexpression group of cell cycle and migration regulators in epithelial cells, but other responses in other cell types (Ikushima et al. 2008), and members of the Ets family of transcription factors and transcription factor activator enhancing-binding protein 2α (TFAP2a; Koinuma et al. 2009). Analysis of Smad2 binding sites in zebrafish early gastrulas furthermore unraveled cooperation with other transcription factors, such as FoxH1, Lef1/β-catenin, Oct1, and Gata6 (Liu et al. 2011). Moreover, comparison of Smad2/3 binding regions in HepG2 hepatoblastoma cells and HaCaT epidermal keratinocytes revealed that 81 % of the binding sites in HepG2 cells are not shared with those in HaCaT cells; however, 32.5 % of the Smad2/3 binding regions overlap with binding sites for hepatocyte nuclear factor 4α (HNF4α) (Mizutani et al. 2011). In addition, genome-wide mapping of Smad3 binding sites revealed that Smad3 cooperates with cell-type-specific master transcription factors, such as Oct4 in embryonic stem cells, Myod1 in myotubes, and PU.1 in pro-B cells (Mullen et al. 2011). Through these mechanisms, the cell-type-specific effects of TGF-β signaling are orchestrated.

Whereas Smad signaling is negatively controlled by ubiquitination and proteasomal degradation of Smad molecules themselves, nuclear Smad signaling can be enhanced by ubiquitination and degradation of transcriptional repressors. Thus, Smad complexes bind the ubiquitin ligase Arkadia which promotes ubiquitination and degradation of the interacting co-repressors Ski and SnoN (Le Scolan et al. 2008; Levy et al. 2007; Nagano et al. 2007), promoting transcription. Because of its ability to enhance the transcriptional activity of Smad, Arkadia acts as a tumor suppressor in colorectal cancer (Sharma et al. 2011). The E3 ligase activity of Arkadia is regulated by the RB1-inducible coiled-coil1 (RB1CC1) protein, which enhances TGF-β signaling by promoting ubiquitination of c-Ski (Koinuma et al. 2011). The level of SnoN is regulated by the anaphase-promoting complex (APC); in response to TGF-β stimulation, casein kinase (CK)II is activated leading to phosphorylation of Cdc27, a key component of APC, which targets SnoN for ubiquitin-mediated degradation (Zhang et al. 2011).

1.13.3 Nuclear Role of I-Smads

The transcriptional roles of R-Smad/Smad4 complexes are well established. However, there are indications that also Smad7 has a nuclear function. Thus, Smad7 interacts with the transcription factor MyoD and antagonizes the repressive effect of the MAP-kinase kinase MEK on MyoD function, thereby promoting myogenic differentiation (Miyake et al. 2010).

1.13.4 Epigenetic Regulation of Smad Signaling

Smad signaling is subject to epigenetic regulation. Thus, the co-repressor TGF-β-induced factor 1 (TGIF1) recruits histone deacetylase activity to Smad2 and thereby represses Smad signaling (Wotton et al. 2001; Wotton et al. 1999a, b). The ubiquitin ligase Fbxw7 targets TGIF1 for degradation and thus enhances the transcriptional activity of Smads (Bengoechea-Alonso and Ericsson 2010). Moreover, TGF-β suppression of the production of interleukin (IL)-2 by T cells involves Smad3-mediated recruitment of the histone H3 K9 methyl transferases Setdb1 and Suv39h1 to the proximal region of the IL-2 gene promoter (Wakabayashi et al. 2011). TIFγ/TRIMM33/ectodermin has a PHD finger-bromodomain which binds histone H3 that is unmethylated at K4 and R2, methylated at K9 and acetylated at K18 and K23; since its ubiquitin ligase activity is induced by histone binding, TIF1γ determines the time Smad complexes remain bound to their promoters by ubiquitination and inactivation of Smad4 (Agricola et al. 2011; Xi et al. 2011).

De-methylation of DNA has also been shown to be essential for regulation of a subset of TGF-β-dependent genes. Thus, TGF-β stimulates active de-methylation of the $p15^{ink4b}$ promoter in a process involving loss of the DNA methyltransferase DNMT3, allowing recruitment of Smad2/3, the CBP acetyltransferase and the DNA glycosylase TDG or the methyl CpG binding domain 4 (MBD4) protein to the same promoter region (reviewed by Thillainadesan et al. 2012).

1.13.5 Regulation of Growth Arrest by Smads

One of the characteristic effects of TGF-β is its ability to inhibit cell proliferation. Smad complexes have key roles in this process by regulating the transcription of genes coding for molecules involved in cell cycle control. Thus, TGF-β suppresses mitogenic signals, e.g. the Myc and Id transcription factors, and induces signals that inhibits the cell cycle, e.g. p15, p21, and p57 (reviewed by Massagué 2004).

1.13.6 Regulation of EMT by Smads

TGF-β is a potent inducer of EMT, i.e. a process during which epithelial cells lose their epithelial characters, such as polarity and cell–cell junctions, and acquire a more mesenchymal phenotype with increased production of matrix molecules, and cytokines and growth factors that stimulate cell migration. EMT is considered important for the invasiveness and metastasis of epithelial tumors. TGF-β regulates EMT by induction of a set of transcription factors, i.e. the basic helix-loop-helix proteins Twist and E47, the Zinc finger proteins Snail and Slug, the Zinc finger and homeodomain proteins ZEB1 and ZEB2, and FOXC2 (reviewed by Heldin et al. 2012). TGF-β induces ubiquitination and proteasomal degradation of the transcription factor Klf4 (Hu and Wan 2011), which is of key importance for the induction of Slug (Liu et al. 2012). Together, the action of these transcription factors leads to repression of E-Cadherin and other epithelial markers and enhancement of the expression of mesenchymal markers such as fibronectin (reviewed by Moustakas and Heldin 2012).

The induction of EMT by TGF-β is enhanced by ZEB1- and ZEB2-mediated downregulation of epithelial splicing regulatory proteins (ESRPs) (Horiguchi et al. 2012). This results in altered splicing of several proteins, including fibroblast growth factor (FGF) receptors 1, 2, and 3 which thereby are converted from the IIIb to the IIIc forms, causing a switch in ligand binding from FGF-7 and -10 to FGF-2 and -4 (Shirakihara et al. 2011; Warzecha et al. 2009).

Posttranscriptional mechanisms also affect TGF-β-induced EMT. Thus, the heterogenous nuclear ribonucleoprotein E1 (hnRNP E1) binds a 33 nucleotide element in the 3′ untranslated regions of the mRNAs of Dab2 and the cytokine interleukin-like EMT inducer (ILEI) and represses their translation. TGF-β induces phosphorylation of hnRNP E1 by Akt2, whereby it is released from the mRNAs which then can be translated, promoting EMT (Chaudhury et al. 2010).

1.14 Non-Smad Signaling

In addition to signaling via R-Smads, there are other signaling pathways activated in TGF-β stimulated cells (Fig. 1.1).

1.14.1 MAP-Kinase Pathways

It has been known for a long time that TGF-β activates the Erk, JNK, and p38 MAP-kinase pathways (Mulder 2000). These pathways have significant roles in TGF-β signaling, as is shown by the observation that TGF-β stimulation of Erk and JNK MAP-kinases drives the formation of aortic aneurysms in Marfan syndrome mice

(Holm et al. 2011). One mechanism of activation of Erk MAP-kinase is via docking of the adaptor protein Shc to the TβRI/TβRII complex, whereby it is phosphorylated on tyrosine residue(s) and become capable of binding the Grb2/Sos1 complex, thus activating the Ras/Erk MAP-kinase pathway (Lee et al. 2007b). On the other hand, TGF-β has been found to selectively activate Erk MAP-kinase in cells with high levels of TβRII, such as dermal cells, but not in cells with low levels of TβRII, such as epidermal cells in a manner which is not dependent on the kinase activity of TβRI (Bandyopadhyay et al. 2011).

Whereas activation of Erk may promote cell proliferation, activation of JNK and p38 MAP-kinases has been implicated in the apoptotic effect of TGF-β. JNK is activated via docking of the adaptor protein Daxx to TβRII (Perlman et al. 2001). Phosphorylation of Daxx by the homeodomain-interacting protein kinase 2 (HIPK2) activates the MAP-kinase kinases MKK4 and MKK7, which ultimately activates JNK (Hofmann et al. 2003). In prostate cancer cells, the p38 MAP-kinase is activated by the binding and activation of the E3 ligase TRAF6 to a motif in the juxtamembrane part of TβRI. TβRI also binds the TGF-β activated kinase (TAK)1 in a Smad7-dependent manner. TRAF6 then performs K63 poly-ubiquitination of TAK1, which thereby is activated, leading to activation of the downstream MKK3 or MKK6 and, finally, p38 (Edlund et al. 2003; Sorrentino et al. 2008; Yamashita et al. 2008). Interestingly, the TRAF6-mediated activation of p38 is independent of the kinase activities of TβRI and TβRII (Sorrentino et al. 2008).

In T cells, the de-ubiquitinase CYLD negatively regulates the activation of TAK1 and p38 (Zhao et al. 2011). Moreover, TGF-β signaling via TAK1 is important for the function of regulatory T cells (Gu et al. 2012). Together, these findings indicate a key role for TAK1 in TGF-β signaling.

1.14.2 Src, PI3-Kinase, and mTOR

In addition to apoptotic pathways, TGF-β activates pro-survival pathways, including the PI3-kinase/Akt pathway TGF-β (Yi et al. 2005). The tyrosine kinase Src has also been shown to be activated by TGF-β and to be important for activation of PI3-kinase (Park et al. 2004; Tanaka et al. 2004). Moreover, TGF-β rapidly induces activation of mTOR complex 1 (mTORC1) and the downstream S6 kinase in a PI3-kinase-dependent manner, leading to increased protein synthesis, cell size, motility, and invasion (Lamouille and Derynck 2007). TGF-β also activates the mTOR complex 2 (mTORC2), which has also been shown to be important for EMT and invasion (Lamouille et al. 2012).

1.14.3 Rho GTPases

TGF-β induces rapid actin reorganization and stress fiber formation by activation of the small GTPases RhoA and RhoB and the downstream effectors ROCK, Lim kinase 2 and cofilin (Vardouli et al. 2005). TGF-β upregulates NET1, a guanine

nucleotide exchange factor for RhoA, in a Smad3-dependent manner (Lee et al. 2010; Shen et al. 2001). In keratinocytes, TGF-β was found to induce another member of the NET1 family, NET1A, in a manner which is dependent both on Smads and Erk MAP-kinase (Papadimitriou et al. 2012). Upon prolonged TGF-β stimulation, NET1A is subject to proteasomal degradation and translational silencing by miR-24, contributing to EMT (Papadimitriou et al. 2012). In prostate cancer cells, TGF-β activates RhoA and Cdc42 leading to reorganization of the actin filament system (Edlund et al. 2002), in a Smad7-dependent manner (Edlund et al. 2004). A mechanism for degradation of RhoA in epithelial cells has been demonstrated. Thus, after TGF-β stimulation, in addition to phosphorylating TβRI, TβRII also phosphorylates the polarity protein PAR6, leading to the recruitment of Smurf1 and subsequent degradation of RhoA contributing to the dissolution of tight junctions (Ozdamar et al. 2005).

1.14.4 Nuclear TβRI

TRAF6 was recently shown to ubiquitinate, in addition to TAK1, also TβRI which makes the receptor susceptible for cleavage by the metalloprotease ADAM17; this liberates the intracellular domain of TβRI which is translocated to the nucleus where it interacts with the co-activator p300 and induces several genes involved in cell migration and invasiveness (Mu et al. 2011). Full-length TβRI has also been found to accumulate in the nucleus under certain conditions. Thus, in ErbB2 transformed cells, which have high amounts of the GTPase Ran that is important for nuclear translocation, TβRI was shown to enter the nucleus in a Smad2/3-dependent manner (Chandra et al. 2012). Nuclear TβRI was found to associate with purine-rich RNA sequences synergistically with the RNA-binding factor hnRNPA1 and may thus affect RNA processing.

1.15 Crosstalk with Other Pathways

TGF-β signaling is modulated by crosstalk with several other signaling pathways, which contributes to the characteristic context-dependency of TGF-β signaling.

1.15.1 Wnt

Wnt is a large family of factors that are implicated in stimulation of cell proliferation during embryonal development and tumorigenesis. Key molecules in the Wnt signaling pathway are the transcription factors β-catenin, T cell factor (TCF), and lymphoid enhancer factor (LEF). Smads form complexes with both LEF1 (Vincent et al. 2009) and β-catenin (Kim et al. 2009; Zhou et al. 2012), which enhance the induction of EMT. In addition, Smad7 forms a complex with β-catenin, which was found to be important for TGF-β-induced apoptosis (Edlund et al. 2005).

1.15.2 Notch

The Notch pathway specifies cell fate determination during development. TGF-β induces several Notch receptor ligands, including Jagged1 (Niimi et al. 2007; Zavadil et al. 2004), and Notch signaling induces TGF-β (Aoyagi-Ikeda et al. 2011). The cooperation between TGF-β and Notch signaling enhances EMT. However, there are reports that in certain cell types, e.g. esophageal epithelial cells, Notch signaling counteracts EMT by induction of miR200 which targets ZEB and TGF-β (Ohashi et al. 2011).

1.15.3 Tyrosine Kinase Receptors

A major pathway induced by tyrosine kinase receptors is the Ras pathway. Cooperation between Ras and TGF-β signaling is particularly important during EMT (Gotzmann et al. 2006). In hepatocarcinoma cells, TGF-β induces both platelet-derived growth factor (PDGF) and PDGF receptors, which enhances PI3-kinase and β-catenin signaling and promotes the survival and invasion of the cancer cells (Fischer et al. 2007). Enhanced PI3-kinase signaling also activates Akt, which phosphorylates and activates Twist, promoting EMT (Xue et al. 2012).

1.15.4 Hippo

The Hippo pathway senses cell density and controls cell growth via the transcriptional regulators TAZ and YAP. TAZ/YAP binds Smad complexes and sequesters them in the cytoplasm in high density cell cultures, thereby attenuating TGF-β signaling (Varelas et al. 2008). Moreover, the Crumbs polarity complex interacts with TAZ/YAP and promotes their phosphorylation and cytoplasmic retention; disruption of the Crumbs complex enhances TGF-β signaling and promotes EMT (Varelas et al. 2010).

1.15.5 Parathyroid Hormone

Parathyroid hormone (PTH) regulates calcium homeostasis and bone metabolism by binding to and activating a G protein-coupled receptor. TβRII forms a complex with and phosphorylates the PTH receptor which modulates the internalization of the receptor complex (Qiu et al. 2010). Through this mechanism TGF-β suppresses PTH signaling.

1.16 Switch in TGF-β Signaling During Tumor Progression

TGF-β acts as a tumor suppressor since it inhibits cell proliferation and induces apoptosis. However, chronic exposure of mammary epithelial NMuMG cells to TGF-β leads to suppression of the anti-proliferative and pro-apoptotic effects of TGF-β and induction of EMT and invasiveness (Gal et al. 2008). Moreover, during tumor progression, TGF-β acquires tumor promoting activities, including promotion of cell invasiveness and metastasis. Whereas the mechanisms behind this switch are not fully understood, some interesting observations have recently been made. Thus, the adaptor protein Dab2, which regulates endocytosis of several receptors, is often downregulated in squamous cell carcinomas, and low levels correlate with poor prognosis (Hannigan et al. 2010). Downregulation of Dab2 blocks TGF-β-mediated cell growth arrest and instead promotes TGF-β-induced cell motility, anchorage-independent growth and tumor growth in vivo.

Another mechanism involves the transcription factor distal-less homeobox 2 (Dlx2), which is upregulated by TGF-β. It attenuates TGF-β-induced growth arrest by downregulating TβRII and promotes cell growth and survival by upregulating the epidermal growth factor family member betacellulin (Yilmaz et al. 2011).

Growing tumors are characterized by hypoxia due to poor vascularization. Smad7 is induced by hypoxia in a hypoxia-inducible factor (HIF)- and von Hippel-Lindau protein (pVHL)-dependent manner (Heikkinen et al. 2010a). Interestingly, the inhibitory effect of Smad7 on TGF-β signaling during normoxic condition is converted to a promoting effect of Smad7 on tumor invasion during hypoxia (Heikkinen et al. 2010b). It has also been observed that Smad7 promotes liver metastases of colorectal tumors (Halder et al. 2008). The activity of HIF is controlled by HIF prolyl hydroxylases (PHDs). Knockdown of PHD2 was found to prevent the switch of TGF-β from being a tumor suppressor to being a tumor promoter (Ameln et al. 2011).

1.17 Future Perspectives

Recent work has given ample examples of mechanisms that control TGF-β signaling on essentially all levels. The fact that such an elaborate machinery has evolved probably reflects the importance of TGF-β signaling during embryogenesis and tissue homeostasis, with the concomitant need to carefully titrate its signaling level. The importance of Smads in TGF-β signaling is well established. The activity of both TGF-β receptors and Smads is controlled by a number of posttranslational modifications, although the list of modifying enzymes and modified amino acid residues is already very long, it is likely that additional modifications will be discovered in the future. The availability of sensitive and accurate mass spectrometry techniques will facilitate the search for additional posttranslational modifications.

In most cells, TGF-β activates both Smad2 and Smad3. Although these molecules are structurally very similar, they have very different effects. Some of the differences can be explained by the fact that Smad3, but not Smad2, binds DNA. However, the detailed mechanism of involvement of Smad2 versus Smad3 in TGF-β signaling remains to be elucidated.

In addition to Smads, a number of other signaling pathways are activated in TGF-β stimulated cells, and a number of pathways activated by other growth factors and cytokines are modulated by TGF-β signaling. An important aim for future research will be to determine the mechanisms whereby such pathways are activated by TGF-β, as well as their importance for the various cellular effects of TGF-β.

A remarkable feature of TGF-β signaling is that it is very context-dependent, i.e. certain responses are seen only in certain cell types and under certain conditions. Some insights into the mechanisms for context-dependency have come from the finding that Smads cooperate with several master regulators of transcription and thus contribute to the establishment of different transcriptional programs in different cell types. However, it is likely that there are additional mechanisms involved in the context-dependence of TGF-β signaling, which remain to be discovered. One functionally important aspect of the context-dependency is the switch of TGF-β signaling from being tumor suppressive to being tumor promoting that occurs during tumor progression. Although some mechanisms explaining this switch have been elucidated, additional work is needed to get a more complete picture.

In conclusion, despite the fact that the TGF-β signaling research field is now becoming rather mature, important questions still remain to be answered.

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Chapter 2
TGF-β Signaling in Stem Cell Fate Determination

Zhongwei Li, Danny Huylebroeck, and Ye-Guang Chen

Abstract Stem cells, which can self-renew and can differentiate to various cell types, have great potential applications in regenerative medicine. The signaling pathways employed by TGF-β family members (including TGF-β, Activin, Nodal, BMPs, and others) are evolutionarily conserved and regulate embryogenesis and adult tissue homeostasis and repair. Disturbance of production or bioactivity of the ligands and of intracellular signal transduction elicited from TGF-β family members (generally called TGF-β signaling) can lead to various diseases, including cancer, fibrosis, and cardiovascular diseases. Consistent with their critical roles in directing normal development, TGF-β family members have been established as key extrinsic signals that regulate fate commitment in both embryonic and adult stem cells. In this chapter, we review the functions and the underlying molecular mechanisms of TGF-β family members in determining embryonic stem cell fate choices between self-renewal, commitment, and subsequent differentiation to three germ layers and their progenies. We also summarize how TGF-β-related factors control fate commitment of five extensively investigated adult stem cells—intestinal, hair follicle, neural, hematopoietic, and mesenchymal stem cells. The principles gained from these studies illustrate on how stem cell fates can be regulated by extrinsic signals and pave a road for the potential medical applications of stem cells, either as transplanted cells after ex vivo expansion or as in vivo resident stem cells activated in endogenous stem cell compartments.

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2.1 Introduction

Stem cells can be derived from both early embryos and various adult tissues. These cells can self-renew and have the ability to differentiate to multiple cell types, thus holding great promise in regenerative medicine. Embryonic stem (ES) cells are derived from the inner cell mass of blastocyst stage embryos (Evans and Kaufman 1981; Martin 1981; Thomson et al. 1998). ES cells can be propagated indefinitely in vitro while maintaining their ability of self-renewal and pluripotency (the ability to differentiate to all three embryonic germ layers and derivatives). Understanding how ES cells sustain self-renewal and differentiate—the ES cell fate determination mechanisms—will pave the road for medical applications of pluripotent stem cells in general. ES cell fates are determined at different levels by extrinsic signals, intrinsic core transcription factors, and epigenetic regulators (Young 2011). Transforming growth factor-β (TGF-β) and related factors play critical roles in embryonic development and in adult tissue homeostasis and repair (Shi and Massague 2003; Massague and Chen 2000). The TGF-β family can be divided into two major groups—the TGF-β/Activin/Nodal and the bone morphogenetic proteins (BMPs)—based amongst others on their signal transduction and regulation mechanisms, which are discussed in depth elsewhere in this book. Briefly, as an extrinsic signal, TGF-β signaling is initiated by binding of the dimeric ligands to the complex of transmembrane receptors, leading to activation of the serine/threonine kinase in the intracellular domain of the type I receptors in the complex. These activated receptors subsequently phosphorylate and activate the cytoplasmic signaling mediators—the R-Smads (Smad1/5/8 for BMP signaling and Smad2/3 for TGF-β/Activin/Nodal signaling), and then the activated R-Smads form a complex with Smad4, the common Smad (co-Smad), and accumulate in the nucleus to directly participate in the regulation of target gene expression (Massague and Chen 2000; Shi and Massague 2003; Feng and Derynck 2005; Heldin et al. 1997; Moustakas et al. 2001). Consistent with its importance in developmental biology, emerging evidence also indicates that TGF-β signaling has profound effects both on the maintenance of ES cell self-renewal state and on the ES cell differentiation into multiple cell lineages. In addition to ES cells, somatic stem cells from different types of tissues have been identified, and they have been documented to play critical roles in tissue homeostasis. TGF-β signaling is also critical in fate determination of these adult stem cells (Watabe and Miyazono 2009; Seuntjens et al. 2009). In this chapter, we summarize and update the current understanding of TGF-β signaling in the fate regulation of ES cells as well as five different kinds of somatic stem cells and discuss their potential medical applications.

2.2 TGF-β and BMP Signaling in ES Cell Fate Determination

2.2.1 BMP Signaling in ES Cell Fate Determination

2.2.1.1 BMP Signaling in ES Cell Self-Renewal

BMP supports the self-renewal state of mouse ES cells. The first definitive evidence for the importance of BMP signaling in mouse ES cell self-renewal was provided by Austin Smith's group in 2003. They found that in feeder- and serum-free N2B27 chemically defined medium (CDM), addition of leukemia inhibitory factor (LIF) and BMP4 can support long-term self-renewal of mouse ES cells (Ying et al. 2003a). In the absence of LIF and BMP4, mouse ES cells differentiate to *Sox1*$^{+}$ neural precursor cells by default (Ying et al. 2003a, b). LIF alone can only partially inhibit neural differentiation and is not sufficient to sustain the ES cell self-renewal, whereas BMP4 alone induces ES differentiation to flat, epithelial-like cells (Ying et al. 2003a, b). Interestingly, in the presence of both LIF and BMP4, mouse ES cell self-renewal is sustained for multiple passages as evidenced by cell morphology, marker gene expression, and chimera contributions (Ying et al. 2003a). Further investigation indicated that transcriptional upregulation of inhibitor of differentiation (*Id*) family genes by BMP signaling mediates some of BMP4's functions and overexpression of *Id1* can bypass the requirement of BMP4 and sustains mouse ES cell self-renewal in cooperation with LIF. *Id* family proteins may inhibit neural differentiation via antagonizing precociously expressed neurogenic DNA-binding basic helix-loop-helix (bHLH) transcriptional activators in mouse ES cells (Ying et al. 2003a) (Fig. 2.1).

To investigate how BMP signaling determines mouse ES cell fate from a genome-wide perspective, Fei et al. explored genomic promoter enrichment of Smad1/5 and Smad4 proteins in mouse ES cells by using a chip-based chromatin immunoprecipitation (ChIP) method (Fei et al. 2010a). They found that Smad1/5 and Smad4 have a large portion of overlapping target genes and these genes include mainly developmental regulators. Their promoters are enriched for both histone H3K27 and H3K4 trimethylation marks (the so-called "bivalent-mark"), which is believed to maintain these genes in the poised state—repressed in self-renewal while rapidly expressed upon differentiation. This co-occupancy pattern suggested that BMP/Smad signaling functions via repressing a large cohort of differentiation-related genes (Fei et al. 2010a). To get a better understanding of the transcriptional regulatory network of external signaling and internal core transcription factors, Chen et al. carried out ChIP-seq to map the locations of 15 transcription factors in mouse ES cells (Chen et al. 2008b). These transcription factors include STAT3 as a mediator of LIF signaling, Smad1 as a mediator of BMP signaling and also factors that play critical roles as ES cell core transcription factors or somatic reprogramming factors. They observed the frequent overlapping binding patterns of STAT3, Smad1, OCT4,

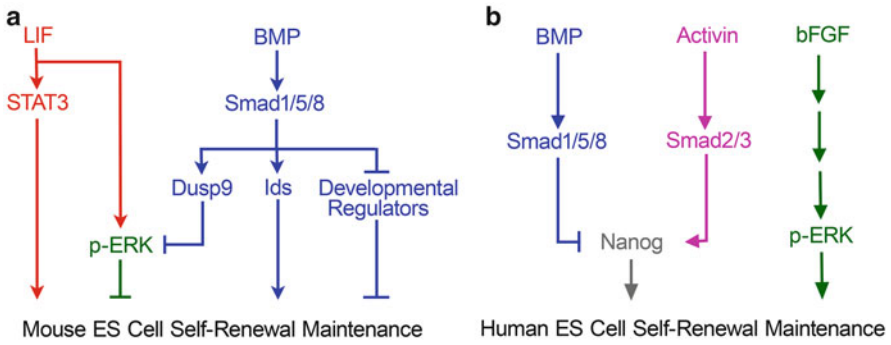

Fig. 2.1 Self-Renewal maintenance of mouse and human ES Cells regulated by TGF-β family members. (**a**) BMP and LIF signaling together maintain mouse ES cell self-renewal. LIF signaling, via activation of downstream STAT3, regulates target gene expression directly and contributes to mouse ES cell self-renewal. The activation of p-ERK1/2 by LIF signaling is suppressed by BMP signaling through transcriptional upregulation of DUSP9. Balanced p-ERK1/2 activity ensures mouse ES cell self-renewal. BMP signaling also enhances mouse ES cell self-renewal by induction of Ids and Nanog and by inhibiting expression of developmental regulators. (**b**) Activin and bFGF signaling support human ES cell self-renewal. Activin upregulates Nanog expression while bFGF mainly exerts its function through ERK1/2 activation. BMP signaling induces robust differentiation, partially by antagonizing Nanog induction by Activin signaling

NANOG, and SOX2, which suggest that external LIF and BMP signaling are connected to the core transcription factor network at the transcriptional level. They also found that the binding of Smad1 and STAT3 in the co-bound sites is dependent on OCT4 binding, suggesting a hierarchy of regulatory interactions between OCT4, Smad1 and STAT3 in mouse ES cells (Chen et al. 2008b).

Recently, a novel mechanism was described which links BMP signaling with ERK activity control for achieving maintenance of self-renewal of cultured mouse ES cells (Li and Chen 2012; Li et al. 2012). ERK is a critical intrinsic regulator to balance self-renewal vs. differentiation of mouse ES cells. High ERK activity triggers mouse ES differentiation (Yoshida-Koide et al. 2004) while low ERK activity sustains self-renewal (Kunath et al. 2007; Stavridis et al. 2007; Ying et al. 2008). Li et al. found that BMP signaling can transcriptionally upregulate dual specificity phosphatase 9 (*Dusp9*), an ERK-preferring phosphatase. The elevated expression of *Dusp9* represses ERK signals and balances the ERK-activating function of LIF signaling, thus contributing to the maintenance of mouse ES cell self-renewal (Li and Chen 2012; Li et al. 2012). Therefore, mouse ES cell self-renewal is sustained by the interplay between extrinsic signals and intrinsic factors. Considering the large amount of downstream target genes of BMP signaling, it is even likely that BMP signaling fully exerts its self-renewal-supportive function through the coordinated regulation of *Id* family genes, *Dusp9*, and the differentiation-related genes.

BMP signaling and LIF signaling may interplay with each other via Nanog (Suzuki et al. 2006). Nanog expression needs both STAT3 and T (Brachyury). STAT3 is activated by LIF while T is induced by Smad1-mediated BMP signaling. Interestingly, Nanog in turn suppresses BMP-induced mesoderm differentiation of mouse ES cells by physically interacting with Smad1.

In complete contrast to the role in mouse ES cells, BMP has been established as a strong differentiation-inducing signal in human ES cells (Fig. 2.1). Several studies have indicated that inhibition of BMP signaling can enhance human ES cell self-renewal (James et al. 2005; Xu et al. 2005; Wang et al. 2005). For feeder-free growth, human ES cells are routinely cultured in medium that contains basic fibroblast growth factor (bFGF, also known as FGF2) and knockout serum replacement (KSR), which is conditioned in the presence of mouse embryonic fibroblasts (MEFs) before feeding hES cells (the so-called "conditioned medium," CM) (Xu et al. 2001). Unconditioned medium (UM) with only bFGF and KSR but without factors secreted from MEFs cannot fully support hES cell self-renewal. It was observed that compared to the ones in CM, human ES cells cultured in UM showed higher p-Smad1/5/8 activity, suggesting activation of BMP signaling may drive human ES cell differentiation (Xu et al. 2005; James et al. 2005). Consistently, addition of high doses of the BMP inhibitor Noggin to UM reduced this p-Smad1/5/8 activation, resulting in long-term self-renewal of pluripotent-state human ES cells (Xu et al. 2005; Wang et al. 2005). It is noteworthy that different from BMP signaling, TGF-β/Activin/Nodal signaling contributes to human ES cell self-renewal maintenance, and this may be achieved in part through antagonizing BMP signaling (James et al. 2005; Wu et al. 2008; Xiao et al. 2006) (Fig. 2.1). Like Noggin, Activin A also inhibited p-Smad1/5/8 activation and hence could provoke sustained human ES cell self-renewal (James et al. 2005). Consistently, inhibition of endogenous TGF-β/Activin/Nodal signaling in CM resulted in trophoblast differentiation, similar to the effect of added BMP4 (Wu et al. 2008). The mutually antagonistic effects of TGF-β/Activin/Nodal signaling and BMP signaling likely converge at the transcriptional regulation of *Nanog* expression. Smad2/3 has been shown to bind to the *Nanog* proximal promoter region in self-renewal-state human ES cells and up-hold *Nanog* mRNA steady-state expression (Xu et al. 2008). BMP4 treatment induces the substitution of Smad2/3 with Smad1/5/8 in this proximal promoter, resulting in a decreased expression of *Nanog* and thus initiating differentiation. This transcriptional regulation is also dependent on the Oct/Sox-binding regulatory elements in the *Nanog* promoter region, indicating the involvement of core transcription factors in this process and again the convergence of external signals and intrinsic regulators on human ES cell fate determination (Xu et al. 2008). The different functions of BMP signal in mouse and human ES cells were proposed to be due to the two different developmental stages that mouse and human ES cells represent—mouse ES cells equivalent to the more primitive inner cell mass of the blastocyst stage while human ES cells resembling the cells in the elongated epiblast stage (Nichols and Smith 2009).

2.2.1.2 BMP Signaling in Extraembryonic Differentiation of ES Cells

In human ES cells cultured in monolayer, activation of BMP signaling drives efficient differentiation to cells of the trophoblast lineage (Xu et al. 2002). Further investigation indicated that differentiation starts from the periphery to the central region of the ES colonies, and this process takes place more efficiently in the

absence of FGF2 and under atmospheric oxygen levels (Das et al. 2007). The trophoblast-inducing activity of BMP signaling is also supported by other studies. For instance, in human ES cell clones deficient in gene expression of *PIG-A* (phosphatidyl-inositol-glycan class A), the cells cannot undergo efficient trophoblast differentiation upon treatment with BMP4 or during spontaneous embryoid body (EB) formation (Chen et al. 2008a). Investigation into the underlying mechanisms revealed that PIG-A deficiency impairs the normal function of GPI-anchored BMP co-receptors. Hence, the lack of full BMP signaling activation may very well lead to the resistance to enter trophoblast differentiation (Chen et al. 2008a). These observations indicate that BMP is a robust trophoblast inducer in human ES cells.

2.2.1.3 BMP Signaling in Germ Cell Differentiation of ES Cells

Primordial germ cells (PGCs) are specified from epiblast cells at the onset of the gastrulation stage. It has been well documented that a group of BMP proteins, including BMP4, BMP7 and BMP8b, and their downstream Smads Smad1 and Smad5, and likely their partner transcription factors, play an inductive role in PGC formation during mouse embryonic development (Zhao 2003; Saitou and Yamaji 2010; Saitou 2009). Consistent with its germ cell-inducing function in vivo (Okamura et al. 2005; Hayashi et al. 2002; Tremblay et al. 2001; Chang and Matzuk 2001), BMP signaling also facilitates germ cell differentiation of ES cells in vitro. Toyooka et al. (2003) generated mouse vasa homolog (*Mvh*, a marker gene for germ cells) knock-in ES cells carrying GFP or LacZ and observed a dramatic increase of MVH+ cells when ES cells were co-cultured in BMP4 expressing cells. Importantly, after purification and transplantation, these MVH^+ cells contributed to testicular tubules and even mature sperm in vivo. Similarly, using GFP knock-in mouse ES cells based on another germ cell marker gene, i.e. *Stella*, Wei et al. (2008) also observed the promoting effect of BMP on germ cell differentiation of ES cells, and this effect was enhanced dose-dependently by treatment with BMP4. BMP signaling also promotes germ cell differentiation in human ES cells. Kee et al. (2006) found that addition of BMP4 to differentiating EBs induced germ-cell specific marker gene expression, and BMP7 and BMP8b showed additive effects when used in combination with BMP4, resulting in dramatically enhanced $VASA^+$ germ cells. These data indicate that BMP signaling enhances germ cell differentiation in both mouse and human ES cells.

2.2.1.4 BMP Signaling in Neural Differentiation of ES Cells

Consistent with its inhibitory role in neural lineage commitment in early embryonic development, BMP signaling inhibits mouse ES cell differentiation to neural precursor cells in a wide variety of in vitro differentiation systems that were used. In the monolayer differentiation condition with N2B27 culture medium, mouse ES cells efficiently differentiate into *Sox1*+ neural precursor cells, and BMP signaling

dramatically inhibits this process, including via *Id* family proteins to repress the precocious expression of neurogenic bHLH transcription factors (Ying et al. 2003a, b), via *Dusp9* to suppress ERK1/2 activity (Li and Chen 2012; Li et al. 2012) and via other downstream target genes such as *Dpysl2* and *Jmjd3* (Fei et al. 2010a). In the PA6 stromal cell co-culture system to induce neural differentiation, treatment with BMP4 in the early phase also inhibits neural differentiation of mouse ES cells (Mizuseki et al. 2003). Interestingly, when BMP4 is added at appropriate doses in a later phase, it causes the differentiation to neural crest cells and dorsalmost CNS cells, showing a time window-dependent effect (Mizuseki et al. 2003). Consistent with this notion, in the EB-derived neural differentiation system, it was found that early exposure to BMP2 inhibits differentiation to neuroectoderm while later treatment of BMP2 in ES-derived neural precursor cells supports further differentiation to neural crest cells (Gossrau et al. 2007). Zhang et al. (2010) further investigated in detail the time windows of neural inhibition by BMP4 in the serum-free EB (SFEB) neural differentiation system and proposed a step-wise neural differentiation model wherein mouse ES cells first differentiate to an epiblast stem cell (EpiSC)-like state, the so-called ES-derived EpiSCs (ESD-EpiSCs), and then ESD-EpiSCs commit to the neural lineage. In the first step, BMP signaling prevents the formation of ESD-EpiSCs, and in the second step BMP signaling inhibits neural commitment and facilitates non-neural differentiation. Interestingly, the functions of BMP signaling are also partially mediated by *Id* family proteins in both steps and by inhibition of ERK activity specifically in the first step. In this SFEB differentiation system, BMP signaling was further found to repress neural differentiation by inhibiting the expression of *Zfp521*, which seems to be essential and sufficient to drive the intrinsic neural differentiation (Kamiya et al. 2011). However, whether *Zfp521* is also a direct target gene of BMP signaling remains to be investigated. Taken together, current evidence supports that BMP signaling inhibits early neural commitment of mouse ES cells via regulation of a cohort of downstream target genes.

BMP signaling also plays key roles in neural commitment of human ES cells. Similar to what has been observed in mouse ES cells, BMP4 represses neural differentiation induced in the PA6 co-culture system (Zeng et al. 2004) and in serum-free suspension culture conditions (Schulz et al. 2004). On the contrary, inhibition of BMP signaling by Noggin (Pera et al. 2004) or by the small molecule receptor kinase inhibitor dorsomorphin (Zhou et al. 2010) facilitates neural commitment. Interestingly, it was observed that dual inhibition of both BMP signaling and TGF-β/Activin/Nodal signaling by Noggin/SB431542 (Chambers et al. 2009) or by dorsomorphin/SB431542 (Morizane et al. 2011) further enhanced the efficiency of neural differentiation. Indeed, inhibition of TGF-β/Activin/Nodal signaling impairs self-renewal of human ES cells and rapidly drives them into transition from self-renewal to differentiation. During the differentiation process, inhibition of BMP signaling and TGF-β/Activin/Nodal signaling represses trophectoderm differentiation and mesendoderm differentiation, respectively. Thus, by blockage of alternative cell lineages, dual inhibition of BMP and TGF-β/Activin/Nodal signaling drives efficient differentiation of human ES cells to the neural lineage (Chambers et al. 2009). It was also reported that additional inhibition of FGF/ERK signaling further enhanced

neural differentiation efficiency (Greber et al. 2011). Thus, in human ES cells, BMP signaling inhibits neural differentiation and further blockage of self-renewal signals from TGF-β/Activin/Nodal signaling and FGF/ERK signaling can drive efficient neural commitment. These findings favor the default model in human ES cell neural differentiation, consistent with the concept established in embryonic development (Weinstein and Hemmati-Brivanlou 1997; Munoz-Sanjuan and Brivanlou 2002).

2.2.1.5 BMP Signaling in ES Cell Differentiation to Mesendoderm and its Derivatives

BMP signaling is well documented to regulate the differentiation of mouse ES cells to mesendoderm (the precursor cells of both mesoderm and definitive endoderm, the counterpart of primitive streak during early mouse development) and its derivatives. In serum-free suspension cultures, treatment with BMP4 can induce posterior primitive streak (PS)-like cells with high expression of the pan-PS marker *Brachyury* (*Bra*) and posterior PS markers *Mesp1* and *Hoxb1* (Nostro et al. 2008). Interestingly, BMP significantly enhances the expression of *Nodal* and *Wnt3*, and the effects of BMP were found to depend on both TGF-β/Activin/Nodal signaling and canonical Wnt signaling, as the addition of SB431542 or DKK1 (Wnt inhibitor) can abolish the expression of these PS marker genes (Nostro et al. 2008). In contrast, Activin A and low concentrations of Wnt3a induce anterior PS-like cells with high expression of *Bra* and *Foxa2*. In this scenario, if BMP4 is also added, the anterior PS marker gene expression was reduced while the posterior marker genes were dramatically induced. These findings highlight a dominant effect of BMP signaling on posterior PS fate commitment (Nostro et al. 2008).

Flk1$^+$ hematopoietic mesoderm can be derived from the PS-like cells and BMP signaling was found to be necessary for this fate commitment in cooperation with TGF-β/Activin/Nodal and Wnt signaling (Nostro et al. 2008). In the EB differentiation system, BMP, Wnt, and Notch signaling were found to be necessary for *Flk1*$^+$ hematopoietic mesoderm differentiation, and the Ets-type transcription factor *Er71* was found to function downstream of these signaling cascades and regulate *Flk1* expression directly (Lee et al. 2008). BMP signaling also functions in the differentiation process from *Flk1*$^+$ mesoderm to blood cells by activating Wnt signaling. Activated Wnt signaling then cooperates with BMP signaling to activate the Cdx-Hox pathway, thus directing the blood cell fate commitment (Lengerke et al. 2008). In the presence of Activin A in serum-free conditions, mouse ES cells can generate endoderm progenitor cells efficiently, and BMP signaling was found to drive hepatic differentiation together with bFGF and Activin (Gouon-Evans et al. 2006). Thus, in mouse ES cells, BMP signaling contributes widely to cell fate commitment processes from mesendoderm to hematopoietic and hepatic derivatives.

Similar promoting functions of BMP in mesendoderm differentiation have been observed in human ES cells. In the spin-EB differentiation system in which EB formation was performed under a centrifugal force, BMP4 first transiently induces PS marker gene expression, and then—with PS gene expression

declining—mesodermal and endodermal marker gene expression are upregulated (Davis et al. 2008). In a serum-free monolayer differentiation system, short-term treatment with BMP4 transiently induced posterior but not anterior PS genes (Zhang et al. 2008). This is consistent with the strong posterior PS patterning effect of BMP signaling on mouse ES cells (Nostro et al. 2008). Interestingly, long-term treatment with BMP4 in the same system dramatically enhanced the expression of genes of the trophoblast lineage (Zhang et al. 2008). The posterior patterning effect of BMP is also supported by another observation that in ectopic expression of β-catenin-induced PS differentiation process the blockage of BMP signaling significantly decreased the expression of posterior PS genes whereas anterior PS gene expression increased (Sumi et al. 2008). Using a chemically defined medium (mTeSR), Yu et al. (2011) observed mesendoderm induction of BMP, which depends on active FGF signaling. They also revealed that FGF signaling via the MEK-ERK cascade prolongs the expression of *Nanog* during BMP-induced differentiation. This sustained *Nanog* expression ensures *bona fide* induction of *Bra* by BMP and thus contributes to efficient mesendoderm differentiation (Yu et al. 2011). Like in mouse ES cells, BMP signaling also contributes to the differentiation of mesoderm derivatives in human ES cells, such as hematopoietic (Pick et al. 2007; Park et al. 2010) and cardiac lineages (Yang et al. 2008; Kattman et al. 2011). Activin signaling has been well documented as a strong inducer of definitive endoderm (D'Amour et al. 2005). Interestingly, it was found that BMP can enhance the definitive endoderm differentiation efficiency in cooperation with Activin, and BMP also plays key roles in the differentiation from definitive endoderm to its progenies. Culture of ES-derived definitive endoderm in the presence of BMP2 and FGF4 efficiently drives hepatic commitment (Cai et al. 2007), inhibition of BMP signaling exerted at the appropriate time is essential to generate insulin-expressing pancreatic cells (Nostro et al. 2011), and dual inhibition of both TGF-β and BMP signaling can generate anterior foregut endoderm from definitive endoderm (Green et al. 2011). Taken together, BMP signaling has broad effects on mesendoderm commitment and on the differentiation to its derivatives in both mouse and human ES cells.

2.2.2 TGF-β/Activin/Nodal Signaling in ES Cell Fate Determination

2.2.2.1 TGF-β/Activin/Nodal Signaling in ES Cell Self-Renewal

TGF-β/Activin/Nodal signaling ensures human ES cell self-renewal. Human ES cells are maintained in the undifferentiated state on MEF-derived feeder cells in the presence of bFGF and KSR. In the absence of feeder cells, human ES cells can be cultured using conditioned medium harvested from feeder cells whereas unconditioned medium will lead to human ES cell differentiation (Xu et al. 2001; Besser 2004; James et al. 2005; Xiao et al. 2006). TGF-β/Activin/Nodal signaling is

dramatically repressed upon differentiation in this unconditioned medium, as evidenced by decreased levels of phosphorylated Smad2/3 (p-Smad2/3) and its downstream target genes *Nodal*, *Lefty-A* and *Lefty-B* (Xiao et al. 2006; Besser 2004; James et al. 2005). Inhibition of TGF-β/Activin/Nodal signaling in conditioned medium drives human ES cell differentiation (Xiao et al. 2006; Besser 2004; James et al. 2005). On the contrary, addition of Activin A in unconditioned medium maintains similar p-Smad2/3 levels as in conditioned medium and can sustain long-term human ES cell self-renewal (Xiao et al. 2006). In a CDM, Activin A and bFGF can cooperatively support human ES cell self-renewal (Vallier et al. 2005). Mechanistically, TGF-β/Activin/Nodal signaling was found to transcriptionally upregulate the expression of *Nanog* in a Smad-dependent manner, thus contributing to self-renewal maintenance (Xu et al. 2008; Vallier et al. 2009; Singh et al. 2012).

From a genome-wide perspective, Smad2/3 can directly bind to multiple pluripotency gene promoters and regulate their expression transcriptionally (Brown et al. 2011). Smad2/3 also share a large proportion of binding sites that are co-bound by OCT4 and NANOG, suggesting cooperation between extrinsic Activin signaling and intrinsic transcription factors for maintaining human ES cells self-renewal (Brown et al. 2011). TGF-β/Activin/Nodal signaling is hierarchically regulated by the WW-domain containing protein TAZ. In human ES cells, TAZ interacts with the Smad complex, facilitates the translocation of the Smad complex from the cytoplasm to the nucleus, and ensures Smad transcriptional activities (Varelas et al. 2008). Conversely, human ES cell self-renewal cannot be maintained when TAZ function is disturbed, due to the inefficiency of TGF-β/Activin/Nodal signaling (Varelas et al. 2008). Interestingly, the functions of TGF-β/Activin/Nodal signaling are quite different in mouse ES cells, where it is not necessary for self-renewal maintenance (James et al. 2005; Fei et al. 2010b). Inhibition of TGF-β/Activin/Nodal signaling by SB431542 (James et al. 2005) or knockdown of Smad2 (Fei et al. 2010b) has no effects on mouse ES cell self-renewal, but TGF-β/Activin/Nodal signaling seems to be important for normal proliferation of mouse ES cells (Ogawa et al. 2007).

2.2.2.2 TGF-β/Activin/Nodal Signaling in ES Cell Differentiation

In mouse ES cells, TGF-β/Activin/Nodal signaling plays critical roles in mesendoderm and subsequent endoderm commitment. In serum-free conditions, treatment with Activin A induces efficient mesendoderm marker gene expression in both adherent and suspension cultures (Tada et al. 2005; Yasunaga et al. 2005; Gadue et al. 2006; Nostro et al. 2008; Fei et al. 2010b). Interestingly, this effect relies on the cooperation with Wnt signaling. Both Activin A and Wnt3a can induce the expression of mesendoderm marker genes. Inhibition of either Activin or Wnt signaling can abolish the differentiation process induced by Wnt3a and Activin A, showing the interdependence of the two signaling pathways in promoting mesendoderm differentiation (Gadue et al. 2006). Furthermore, Activin A has been shown to induce *Wnt3* expression, while Wnt can upregulate *Nodal* (Gadue et al. 2006).

Using a genome-wide approach, Fei et al. investigated how Smad2 mediates the induction of mesendoderm by Activin and identified Smad2 binding sites in promoter regions across the genome (Fei et al. 2010b). Smad2 binds to promoters of a large number of development-related genes, and one of the Activin/Smad2-induced genes, *Tapbp*, was found to partially mediate the function of Activin/Smad signaling in mesendoderm differentiation (Fei et al. 2010b). Mesendoderm is a bi-potent state that can further generate mesoderm and endoderm derivatives. Continuous Activin signaling has been implicated as necessary for definitive endoderm differentiation from mesendoderm (Yasunaga et al. 2005; Gadue et al. 2006), and the cooperation of Activin, Wnt and BMP signaling drives efficient differentiation of mesendoderm to *Flk1*$^{+}$ mesodermal cells (Nostro et al. 2008), i.e. hematopoietic and cardiac progenitor cells. TGF-β/Activin/Nodal signaling has conserved functions in human ES cells in inducing mesendoderm and subsequent definitive endoderm (D'Amour et al. 2005; McLean et al. 2007; Singh et al. 2012; Sumi et al. 2008) and is also implicated in inhibiting neuroectoderm differentiation (Vallier et al. 2004; Smith et al. 2008).

2.3 TGF-β and BMP Signaling in Adult Stem Cell Fate Determination

TGF-β family members, particularly BMPs, play critical roles in self-renewal and differentiation of adult stem cells. Here, we summarize the current understanding on cell fate determination of intestinal, hair follicle, neural, hematopoietic and mesenchymal stem cells (MSCs) by BMPs, as selected examples (Fig. 2.2).

2.3.1 Intestinal Stem Cell Fate Determination

Intestinal epithelium is vigorously and constantly renewed from a small number of adult stem cells—the intestinal stem cells (ISCs). These ISCs are located at the fourth or fifth cell position from the base of each of the many intestinal crypts ("+4" ISCs) and can generate the entire crypt-villus structure, giving rise to absorptive enterocytes, mucin-producing goblet cells, hormone-secreting enteroendocrine cells and exocrine Paneth cells (van der Flier and Clevers 2009; Scoville et al. 2008). For maintaining intestinal homeostasis, ISC self-renewal and differentiation must be precisely regulated. Multiple signaling pathways in the ISC niche have been identified as critical in this process, including Wnt, Notch, and BMP signaling (van der Flier and Clevers 2009; Scoville et al. 2008). BMP4 is expressed in the intravillus mesenchyme whereas Noggin, the antagonist which can bind and inactivate BMP4, is synthesized in and secreted from cells that are adjacent to the bottom of the crypt and around the ISCs (He et al. 2004). Furthermore, the BMP type I receptor BMPR1A exhibits a graded distribution along the crypt-villus, with ISCs displaying the highest level (He et al. 2004). Conditional gene inactivation of BMPR1A in the intestine (He et al. 2004) or transgenic expression of Noggin (Haramis et al. 2004)

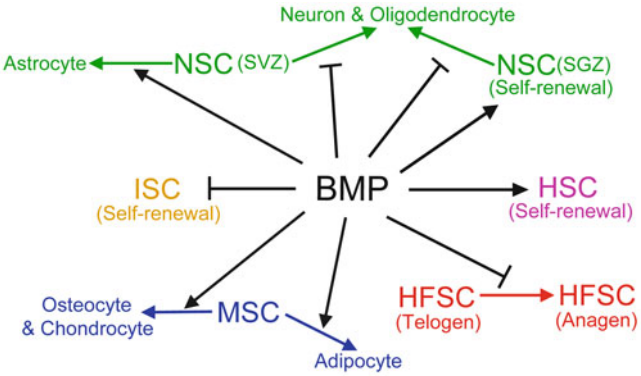

Fig. 2.2 BMP signaling on adult stem cell fate determination. BMP signaling sustains hematopoietic stem cell (HSC) self-renewal while inhibiting intestinal stem cell (ISC) self-renewal. The BMP signal barrier should be overcome for hair follicle stem cells (HFSCs) to transit from the telogen phase to the anagen phase and thus enhancing generation of new hair follicles. BMP signaling also directs mesenchymal stem cell (MSC) differentiation to osteocyte, chondrocyte and adipocyte lineages. Self-renewal of neural stem cells (NSCs) in SGZ is supported by BMP signaling whereas neurogenesis is suppressed. In NSCs from SVZ, BMP signaling induces astrocyte differentiation, at the expense of alternative commitment to neuron and oligodendrocyte cell fates

leads to the formation of ectopic polyps similar to what is seen in human juvenile polyposis syndrome. Further analysis indicated that expansion of stem and progenitor cell populations is responsible for the development of the polyps and that inhibition of BMP signaling by the PI3K-PTEN-AKT pathway enhanced the nuclear β-catenin level and promoted ectopic stem cell proliferation (He et al. 2004). Although BMP signaling plays a negative role on ISC maintenance, it seems to be important for differentiation of ISCs to mature intestinal cell types as the intestinal epithelium-specific knockout of BMPRIA resulted in defects in goblet, enteroendocrine, and Paneth cell maturation (Auclair et al. 2007). Recently, the cycling columnar cells in the crypt base, which are marked by the Wnt target gene Lgr5, have been identified as a novel population of ISCs (Barker et al. 2007). These stem cells can generate crypts-villus structures in vitro, in which $Lgr5^+$ ISCs rapidly self-renew and give rise to all types of intestinal epithelial cells (Sato et al. 2009), including "+4" ISCs (Yan et al. 2012). The presence of Noggin in the culture system is essential for the long-term maintenance of $Lgr5^+$ ISCs, indicating inhibition of BMP signaling is also required for maintenance of $Lgr5^+$ ISCs (Sato et al. 2009).

2.3.2 Hair Follicle Stem Cell Fate Determination

In the adult, hair follicles (HFs) undergo continuous and synchronized cycles to regenerate novel follicles (Blanpain and Fuchs 2009). Such cycle consists of three phases: telogen, the quiescence phase which lasts for months with quiescent hair

follicle stem cells (HFSCs) residing in the bulge; anagen, the regeneration phase when HFSCs are activated and generate novel hair follicles; and catagen, the degradation phase which includes apoptotic retraction (Blanpain and Fuchs 2009). In the telogen phase, BMP mRNAs are expressed by dermal cells, including fibroblasts, adipocytes, and dermal papillae (Plikus et al. 2008). BMP ensures the stem cells to stay quiescent (Horsley et al. 2008). This effect of BMP is partially mediated by directly inducing the NFATc1-encoding gene, which subsequently suppresses *Cdk4* and thus leads to maintenance of stem cell quiescence (Horsley et al. 2008). When follicle stem cells are activated, NFATc1 gene expression is downregulated, leading to a high level of *Cdk4* and thus promoting cell proliferation (Horsley et al. 2008). BMP signaling should be suppressed in the transition from the telogen phase to the anagen phase to enable the successful transition (Horsley et al. 2008; Blessing et al. 1993; Botchkarev et al. 1999, 2001). Ectopic expression of *Bmp4* or gene inactivation of *Noggin* leads to a dramatic delay in hair follicle induction (Blessing et al. 1993; Botchkarev et al. 1999), whereas overexpression of *Noggin* induces transition to the anagen phase (Botchkarev et al. 1999, 2001). Inhibition of BMP signaling may contribute to the anagen transition partially by activation of β-catenin signaling via regulation of PI3K-AKT activity (Zhang et al. 2006), resembling the situation in ISCs (He et al. 2004). In an RNAi screen, *Tbx1* was identified to be critical for long-term HFSC regeneration. Interestingly, *Tbx1* exerts its functions mainly by enhancing the sensitivity of the cells to BMP, further highlighting the gatekeeper role of BMP signaling in HFSC maintenance (Chen et al. 2012). Recently, TGF-β2 was found to be a critical paracrine factor secreted from dermal papillae, and via Smad2/3 signaling, to regulate the expression of *Tmeff1*, which subsequently leads to inhibition of BMP signaling and thereby facilitates the transition from telogen to anagen (Oshimori and Fuchs 2012).

2.3.3 Neural Stem Cell Fate Determination

Neural stem cells (NSCs) are defined as progenitor cells that can generate neural tissues—neurons and glial cells (astrocytes and oligodendrocytes) while at the same time retaining some capacity to self-renew (Gage 2000; Temple 2001). In the adult brain, NSCs mainly reside in two specialized areas: the subventricular zone (SVZ) and the hippocampal subgranular zone (SGZ). NSCs in the SGZ divide infrequently and are mainly maintained in a quiescent state. BMP signaling is active in these NSCs in mice as its blockage by in vivo infusion of Noggin or conditional ablation of *Bmpr1a* or *Smad4* drives these cells into the cell cycle and increased neurogenesis (Mira et al. 2010). However, in the long run, conditional deletion of *Bmpr1a* impaired stem cell division, and precursor cells and newborn neurons were lost, indicating loss of stem cell activity (Mira et al. 2010).

In the case of neural stem cells in the SVZ, BMP signaling specifies differentiation to astrocytes while repressing neuron and oligodendrocyte commitment (Lim et al. 2000; Gomes et al. 2003; Samanta and Kessler 2004; Nakashima et al. 2001;

Kohyama et al. 2010). Although intact BMP signaling components are present in SVZ cells, the adjacent ependymal cells produce Noggin and inhibit BMP signaling, thus providing a niche that enables neurogenesis (Lim et al. 2000). Consistently, in vivo ectopic expression of *Bmp4* resulted in a significant increase of astrocytes and decrease of oligodendrocytes (Gomes et al. 2003), and in various in vitro cultures, the robust astrocyte induction by BMP signaling has been established and shown to occur at the expense of neuron and oligodendrocyte fates (Samanta and Kessler 2004; Nakashima et al. 2001; Kohyama et al. 2010). Target genes of BMP signaling mediate these functions. *Id1*, *Id3*, and *Rest* were indicated to interfere with neurogenic bHLH transcription factors to inhibit neurogenesis (Nakashima et al. 2001; Kohyama et al. 2010), while *Id2* and *Id4* were found to complex with and subsequently repress bHLH transcription factors that are required for oligodendrocyte differentiation (Samanta and Kessler 2004). Thus, by coordinated functions of its direct target genes, BMP signaling restricts alternative fates of neuron and oligodendrocyte commitment from SVZ neural stem cells and directs efficient astrocyte differentiation. In contrast, there is also evidence showing that conditional deletion of Smad4 in the SVZ neural stem cells or infusion of Noggin led to the impairment of neurogenesis and increased the number of oligodentrocytes (Colak et al. 2008). These observations add complexity to the physiological roles of BMP signaling in determining the cell fates. Probably the intensities of BMP signaling in this context may explain the discrepancy, which will be interesting for future study. Using the cultures of fetal neuroepithelial cells which contain neural precursors, Nakashima et al. also observed the synergistic effect between BMP and LIF in inducing astrocyte differentiation. Mechanistically, upon cytokine stimulation, p300 bridges the interaction between Smad1 and STAT3, and the Smad1-STAT3-p300 complex thereafter drives the transcription of the astrocyte marker gene—glial fibrillary acidic protein (GFAP) (Nakashima et al. 1999). Apart from BMP signaling, TGF-β signaling is also involved in NSC biology. Conditional inactivation of *Tgfbr2* results in neuroepithelial cell expansion in the dorsal caudal midbrain and in increased sphere formation and self-renewal of neuroepithelial stem cells. Decreased cell-cycle exit and shortened cell-cycle length are responsible for these phenotypes, and FGF and Wnt signaling were implicated to mediate TGF-β signaling functions, indicating the crosstalk among these signal pathways for coordinating NSC functions appropriately (Falk et al. 2008).

2.3.4 Hematopoietic Stem Cell Fate Determination

Hematopoietic stem cells (HSCs) reside in the bone marrow (BM) of the adult body. HSCs are rare in the BM and they stay at the top of a hierarchy of progenitor blood lineages, capable of reconstituting the whole blood system of a recipient, including lymphocytes (T and B cells), myeloid cells (monocytes and neutrophils), megakaryocytes/platelets, and red blood cells (Orkin 2000; Orkin and Zon 2008). Both BMP and TGF-β signaling are involved in the proliferation and fate control of HSCs. BMP

has been identified as a critical factor to regulate the commitment from mesoderm to hematopoietic progenitor cells in embryonic development from various species (Huber et al. 1998; Maeno et al. 1996; Winnier et al. 1995; Dzierzak and Speck 2008) and has meanwhile also been employed to obtain blood lineage cells from ES cells and from induced pluripotent cells (iPSCs) (Nostro et al. 2008; Pearson et al. 2008; Irion et al. 2010; Chadwick et al. 2003; Park et al. 2010). In in vitro cultured HSCs, high concentrations of BMP4 extend the time of repopulation ability whereas low concentrations of BMP4 induce proliferation and differentiation (Bhatia et al. 1999). To investigate the functions of BMP4 in vivo in the HSC niche, Goldman et al. employed BMP4 hypomorph mice in which mature BMP4 ligands were dramatically decreased in specific tissues due to the S2G point mutation ($Bmp^{S2G/S2G}$) (Goldman et al. 2009). They found that BMP4 is present in various types of cells that constitute the HSC niche, including osteoblasts, endothelial cells, and megakaryocytes. In bone marrow, BMP4 levels were much lower in $Bmp^{S2G/S2G}$ than wild-type mice, and the HSC-enriched c-kit$^+$, Sca-1$^+$, Lineage$^-$ (KSL) cell number was dramatically lower in $Bmp^{S2G/S2G}$ mice. When wild-type HSCs were transplanted to BMP4-deficient recipient mice, the HSC activity was greatly reduced (Goldman et al. 2009). These observations suggest the essential functions of paracrine BMP4 effects in HSC homeostasis. However, Singbrant et al. generated conditional *Smad1*/*Smad5* double knockout mice and found that the BM cells from these adult mice showed normal reconstitution capabilities after transplantation as compared to wild-type mice, arguing against the observations made in the BMP4 polymorph mice (Singbrant et al. 2010). Explanations to this discrepancy include that downstream non-Smad signaling may be involved in mediating BMP4 functions (Zhang 2009) or Smad redundancies may account for the mild phenotype in adult hematopoiesis.

TGF-β signaling has been shown to be a potent inhibitor for HSC proliferation (Sitnicka et al. 1996; Batard et al. 2000), and it is regarded as a key extrinsic signal that keeps HSC quiescent. Cyclin-dependent kinase inhibitors, including *p21* and *p57*, are implicated to mediate TGF-β functions in this process (Ducos et al. 2000; Scandura et al. 2004). Apart from inhibiting HSC proliferation, TGF-β signaling also promotes erythroid differentiation (hemoglobin + cells) in various in vitro studies (Shiozaki et al. 1998; Schwall and Lai 1991; Okafuji et al. 1995; Liu et al. 2000). It has been reported that the different functions of TGF-β can be achieved by distinct Smad complexes as signaling mediators—the classic Smad2/3-Smad4 complex mediates the antiproliferative response while Smad2/3-TIF1γ complex is necessary for erythroid differentiation (He et al. 2006). However, when TGF-β receptor *Tgfbr1* was conditionally knocked out, no abnormal phenotypes were observed for HSCs (Larsson et al. 2005), in contrast to the in vitro inhibitory functions of TGF-β. Moreover, ectopic expression of Smad7, which antagonizes both TGF-β and BMP signaling, even enhanced the self-renewal of HSCs in vivo (Blank et al. 2006). Conversely, in a *Tgfbr2* conditional knockout mouse model, HSC activity was severely impaired (Yamazaki et al. 2011), similar to that observed in conditional knockout of *Smad4* (Karlsson et al. 2007). Thus, whether TGF-β signaling is an essential negative regulator in HSC proliferation is still unclear in a physiological context, and further investigations are needed to fully characterize the functions of TGF-β signaling in HSC functions and homeostasis in vivo.

2.3.5 MSCs Fate Determination

MSCs are multipotent stem cells isolated mainly from adult bone marrow, which can maintain multilineage potential in vitro and differentiate to mesenchymal tissues, including bone, cartilage, fat, muscle, and marrow stroma (Pittenger et al. 1999; Jiang et al. 2002; Barry and Murphy 2004). The proliferation and differentiation of MSCs are regulated by TGF-β family members, often in cooperation with other signaling pathways. Treatment with TGF-β1 (Jian et al. 2006) can promote proliferation of MSCs whereas it will inhibit osteogenic differentiation. Further investigation indicated that TGF-β1 induces nuclear accumulation of β-catenin in MSCs via the physical interaction of the latter with Smad3 (Jian et al. 2006). Consistently, inhibition of TGF-β signaling by SB431542 induces osteoblastic maturation and the anti-osteoblastic function of TGF-β signaling is mediated by inhibitory Smads to antagonize BMP signaling (Maeda et al. 2004). BMP, however, inhibits MSC proliferation and induces osteogenic and chondrogenic differentiation (Partridge et al. 2002; Tsuda et al. 2003; Schmitt et al. 2003; Shea et al. 2003; Noel et al. 2004; Lavery et al. 2008; Liu et al. 2006). Interestingly, BMP signaling also converges to Wnt signaling to exert its anti-proliferative function. In the presence of BMP2, the interaction of Smad1 and Dvl-1 is increased, leading to decreased β-catenin levels in the nucleus and restricted β-catenin activity, thus inhibiting MSC proliferation (Liu et al. 2006). Thus, balanced TGF-β and BMP signaling dictates MSC fates. Bone remodeling requires coordinated bone resorption and subsequent bone formation. Active TGF-β1 is released from osteoclasts upon bone resorption and stimulates the migration of MSCs to the resorptive sites, thus contributing to bone formation (Tang et al. 2009).

Adipose tissues control energy balance, with white adipose tissue storing triglyceride and brown adipose tissue specialized for energy expenditure. Both white and brown adipose tissues can be generated from MSCs, and BMPs play dominant roles in this process. BMP2 and BMP4 specify MSCs to white adipose tissue (Wang et al. 1993; Tang et al. 2004; Jin et al. 2006). By the cooperation of Smad1/4,C/EBPα and Shn-2, BMP2 upregulates the transcription of PPARγ2, the key transcription factor for adipogenesis, to contribute to the white adipose commitment (Jin et al. 2006). BMP7, however, is required for brown fat adipose differentiation in vivo (Loncar 1992; Kalluri and Zeisberg 2003), and multiple lines of evidence also indicate its inductive role in vitro (Tang et al. 2004; Tseng et al. 2008; Schulz and Tseng 2009). A recent study examined the mechanisms and found that BMP7 induces the MSC commitment to brown adipose tissues by activating a series of regulators of the brown adipose fate via the p38 MAPK and PGC-1 pathways (Tseng et al. 2008).

2.4 Perspectives

During embryogenesis BMP signaling regulates multiple lineage specification processes, including neural inhibition, mesoderm commitment, and hematopoietic and cardiac development. Consistent with the in vivo functions in development, BMP

retains these activities in cell fate determination of in vitro cultured pluripotent embryonic stem cells (ES cells), making these cells a great and as it turns out a robust system to investigate the detailed molecular mechanisms that are more difficult to study in embryos-, especially in human embryos. It is also important to note that TGF-β and BMP have been found to function differently in mouse vs. human ES cells. In mouse ES cells, BMP signaling promotes self-renewal by restricting neural differentiation whereas in human ES cells BMP signaling has strong differentiation-initiating effects. This functional difference is likely due to the different developmental stages mouse and human ES cells represent. Indeed, human ES cells resemble more the elongated epiblast stage whereas mouse ES cells are derived from prospective epiblast cells from the inner cell mass of the blastocyst stage and represent a more primitive "naive" state. In contrast to the opposite effects on self-renewal, BMP signaling exerts similar functions in differentiation of mouse and human ES cells.

Both TGF-β and BMP signaling act in a context-dependent manner in stem cell fate determination. For example, BMP signaling sustains mouse ES cell self-renewal in the presence of LIF signaling, while it also promotes differentiation to multiple other lineages in the presence of other extrinsic signals but without LIF. How cells read TGF-β or BMP signals and react accordingly under different scenarios is interesting but as yet not well understood. Three types of contextual determinants were proposed to explain this—the difference of signaling intensity in different contexts, the involvement of different transcriptional cofactors in specific contexts and the different states of epigenetic landscapes that pre-determine the access of Smad proteins to specific genes (for more details, see Massague 2012). These mechanisms may individually or cooperatively mediate the context-dependent effects of TGF-β and BMP signaling in different conditions.

TGF-β family members dictate both embryonic stem and adult stem cell fate specification in cooperation with several other signaling pathways, such as FGF and Wnt signaling. For example, in different signaling contexts, BMP signaling can exert different effects and direct differentiation to different lineages: the FGF-MEK-ERK cascade can shift BMP functions from initiating trophoblast lineage to inducing mesendoderm cells. Different signaling inputs may converge on the transcriptional regulation of downstream target genes or directly fine-tune the intensity and duration of critical signaling. However, how TGF-β and BMP signaling, respectively, integrate with other signaling systems to determine stem cell fates is still an important topic of future research. The intensified application of transcriptomics as well as proteomics (with emphasis on protein–protein interaction) and ChIP-sequencing (protein–DNA interaction) will contribute significantly to identifying the underlying molecular mechanisms of the crosstalk between different signaling pathways, and how extrinsic signaling directs stem cell commitment to specified cell types.

Dissecting the underlying mechanisms of how TGF-β and BMP signaling determine stem cell fates is fundamental to the understanding of stem cell biology, and more importantly, the knowledge obtained from these research could lay the basis for regenerative medicine. The ultimate goal of regenerative medicine is to replace malfunctioned cells/tissues/organs with the good ones. Thus, the most urgent task is

to find ways to direct stem cell differentiation to specific cell types. TGF-β and BMP signaling, whose roles have been well established in embryogenesis, are also found to be key players in a broad spectrum of stem cell fate decision. Considering the convenience to manipulate these signaling (with various ligands, antagonists, chemical inhibitors) and the advantages of signaling-mediated differentiation (virus-free and higher efficiency) compared to the transcription factors-mediated ones, undoubtedly TGF-β and BMP signaling will contribute to future applications of stem cells in regenerative medicine.

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Chapter 3
Emerging Roles of TGF-β Co-receptors in Human Disease

Alison E. Meyer, Karthikeyan Mythreye, and Gerard C. Blobe

Abstract TGF-β signaling is both regulated and mediated by signaling co-receptors. Several TGF-β co-receptors have been identified including endoglin (CD105), the type III TGF-β receptor (TβRIII, betaglycan), neuropilin-1/2, syndecan-2, CD109, and LRP1. These co-receptors serve diverse functions including the regulation of ligand access to other TGF-β receptors and receptor trafficking. The TGF-β co-receptors can also signal directly. The TGF-β co-receptors are broadly expressed, have essential roles in embryonic development, and are frequently altered during disease progression. TGF-β co-receptors regulate cancer initiation and progression through effects on cell growth, migration, invasion, proliferation, and angiogenesis. In addition to their roles in cancer, these co-receptors are dysregulated during development, in vascular disease and fibrotic disorders. Collectively, the TGF-β co-receptors influence disease biology through complex mechanisms involving the regulation of growth factor-dependent and independent signaling events as well as through interactions with diverse scaffolding protein partners.

Keywords Angiogenesis • Betaglycan • Cancer • Co-receptors • Development • Disease • Endoglin • Fibrosis • Neuropilin • Syndecan • TGF-β • TβRIII

3.1 Introduction

TGF-β signaling pathways have essential roles in multiple cellular processes including proliferation, differentiation, and apoptosis (Massague 1998). While canonical TGF-β signaling is mediated by the type I and type II TGF-β receptors, TGF-β

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signaling is both regulated and mediated by signaling co-receptors. Multiple TGF-β co-receptors have been identified including endoglin (CD105) (Cheifetz et al. 1992), the type III TGF-β receptor (TβRIII, betaglycan) (Andres et al. 1991; Lopez-Casillas et al. 1991; Wang et al. 1991), neuropilin-1/2 (Glinka and Prud'homme 2008), syndecan-2 (Chen et al. 2004b), CD109 (Finnson et al. 2006), and LRP1 (Cabello-Verrugio and Brandan 2007). These co-receptors serve diverse functions including the regulation of ligand access to other TGF-β receptors and receptor trafficking. The co-receptors can also signal directly. The TGF-β co-receptors are broadly expressed, have essential roles in embryonic development, and are frequently altered during disease progression (Maring et al. 2012; Bernabeu et al. 2009; Staton et al. 2007; Li et al. 1999; Wild et al. 2012; Stenvers et al. 2003; Theocharis et al. 2010; Gatza et al. 2010). Here we summarize the emerging roles of TGF-β co-receptors in human disease with a focus on endoglin, TβRIII, neuropilin-1/2, and syndecan-2.

3.1.1 Structural Features and Ligand Binding Properties of TGF-β Co-receptors

Structurally, endoglin, TβRIII, neuropilin-1/2, and syndecan-2 all have a large extracellular domain, a short, single-pass transmembrane region, and a short cytoplasmic domain (Essner et al. 2006; Gougos et al. 1992; Mythreye and Blobe 2009a; Lopez-Casillas et al. 1991; Wild et al. 2012). The extracellular domains of TβRIII and syndecan-2 are modified with glycosaminoglycan chains. The extracellular domains of all co-receptors interact with multiple ligand classes (see Table 3.1). Furthermore, these co-receptors all undergo ectodomain shedding, with proteolytic cleavage releasing their soluble extracellular domains from the cell surface. The soluble ectodomains have diverse functions including sequestering ligand to inhibit signaling, binding ligand to facilitate signaling in *trans*, and potentially functioning as ligands themselves (Essner et al. 2006; Gatza et al. 2010; Bernabeu et al. 2009; Wild et al. 2012).

3.1.2 TGF-β Co-receptor Function

Endoglin, which is expressed predominantly in proliferating vascular endothelial cells and smooth muscle cells, has a critical role in angiogenesis (Bourdeau et al. 1999; Fonsatti et al. 2003). Endoglin null mice are embryonic lethal due to defects in vascular development (Li et al. 1999), while mutations of the endoglin gene, ENG, cause the autosomal dominant vascular disease, hereditary hemorrhagic telangiectasia (HHT; discussed in detail below) (McAllister et al. 1994). In endothelial cells, endoglin promotes proliferation through the endothelial-specific type I TGF-β receptor ALK1 and the Smad 1/5/8 pathway, while inhibiting proliferation through

Table 3.1 Ligand specificity of TGF-β co-receptors

Co-receptor	Ligand specificity	References
Endoglin	TGF-β1,3 ActivinA BMP 2,7,9 and 10	Bernabeu et al. (2009), Castonguay et al. (2011)
TβRIII (betaglycan)	TGF-β1, 2 and 3 Inhibin BMP 2, 4, 7 GDF-5 FGF2	Gatza et al. (2010)
Neuropilins 1/2	TGF-β1 Sema3A, 3F VEGF HGF FGF PDGF Shh	Wild et al. (2012)
Syndecan-2	TGF-β1 FGF VEGF PDGF	Essner et al. (2006), Chen et al. (2004b)

BMP bone morphogenetic protein, *FGF* fibroblast growth factor, *GDF* growth/differentiation factor, *HGF* hepatocyte growth factor, *PDGF* platelet-derived growth factor, *Shh* sonic hedgehog, *TGF-β* transforming growth factor-β, *VEGF* vascular endothelial growth factor

the canonical type I TGF-β receptor ALK5 and the Smad2/3 pathway (Goumans et al. 2002). Endoglin has also been shown to participate in Erk (Lee and Blobe 2007), H-Ras (Santibanez et al. 2010), and PI3K/Akt signaling (Lee et al. 2012). Despite its predominant role in angiogenesis, Endoglin is also expressed in some non-endothelial cell types (see Cancer section below). However, the signaling pathways that endoglin utilizes in these cell types have not been well characterized.

TβRIII is an important regulator of cell migration, invasion, growth, and angiogenesis (Gatza et al. 2010). TβRIII null mice are embryonic lethal and exhibit liver and heart defects (Stenvers et al. 2003). TβRIII mediates ligand presentation, whereby ligand is transferred from TβRIII to TβRII to potentiate signaling (Lopez-Casillas et al. 1991; Lopez-Casillas et al. 1993; Wang et al. 1991). In addition to facilitating signaling through the canonical Smad pathways, TβRIII regulates NFκB (Criswell and Arteaga 2007; You et al. 2009) , p38 (Santander and Brandan 2006), and Cdc42 signaling (Mythreye and Blobe 2009b).

Neuropilin-1/2 are vertebrate-specific proteins that are essential for the normal embryological development of the nervous and cardiovascular systems (Staton et al. 2007). Neuropilin-1/2 also regulate angiogenesis, with neuropilin-1 predominating in arterial endothelial cells and neuropilin-2 predominating in vein and lymphatic endothelial cells (Staton et al. 2007). Upon ligand binding, neuropilin-1/2 signal through the p38, Akt, focal adhesion kinase (FAK), and MAPK pathways to regulate numerous diverse processes including proliferation, survival, migration, adhesion, and vascular permeability (Wild et al. 2012).

Syndecan-2 has roles in numerous processes including adhesion, cytoskeletal organization, vesicle transport, synaptic transmission, and axonal migration (Essner et al. 2006). This co-receptor regulates binding to the extracellular matrix (ECM) components laminin, collagen, and fibronectin and can also regulate actin dynamics and FAK (Essner et al. 2006).

3.1.3 Co-receptor Interacting Proteins

While their extracellular domains largely mediate ligand binding, the cytoplasmic domains of the TGF-β co-receptors interact with numerous scaffold proteins to regulate receptor trafficking and signaling. Interestingly, endoglin, TβRIII, neuropilin-1/2, and syndecan-2 all interact with the PDZ domain-containing protein GIPC (GAIP-interacting protein C-terminus; also known as synectin or NIP) (Essner et al. 2006; Wang et al. 2006; Blobe et al. 2001; Lee et al. 2008; Cai and Reed 1999). In the case of neuropilin-1/2, this interaction is important for proper arterial branching morphogenesis and can promote the internalization of integrin to regulate cell adhesion to fibronectin (Chittenden et al. 2006; Valdembri et al. 2009). In contrast to integrin, both endoglin and TβRIII are stabilized on the cell surface through their interaction with GIPC (Blobe et al. 2001; Lee et al. 2008). In addition to GIPC, endoglin and TβRIII can associate with β-arrestin2, resulting in their internalization (Chen et al. 2003; Lee and Blobe 2007). Endoglin can also interact with zyxin (Conley et al. 2004) and zyxin-related protein 1 (Sanz-Rodriguez et al. 2004) to regulate cell adhesion, as well as with the cytosolic dynein light chain family member Tctex2β (Meng et al. 2006), which links endoglin with the microtubule-based transport machinery. Interestingly, the cytoplasmic domain of syndecan-2 has been shown to specifically associate with TβRIII, although the consequences of this interaction are unknown (Chen et al. 2004b). Syndecan-2 can also interact with the cell adhesion molecule fibronectin, α5β1 integrin, the PDZ domain proteins GIPC, CASK/LIN-2, synbindin, and syntenin, and the ERM family proteins ezrin, radixin, and moesin, with most of these interactions facilitating actin reorganization and cell-substrate adhesion (Essner et al. 2006).

3.2 TGF-β Co-receptors in Cancer

In normal epithelial cells and in the early stages of tumorigenesis, TGF-β acts as a tumor suppressor by inhibiting growth and inducing apoptosis or differentiation, when appropriate (Elliott and Blobe 2005). However, during cancer progression, cancer cells lose this inhibitory growth response and respond instead with increased migration and invasion (Elliott and Blobe 2005). TGF-β also functions to promote cancer progression by inhibiting the immune system and promoting angiogenesis (Elliott and Blobe 2005). Much like TGF-β ligands, the co-receptors regulate both

cancer initiation and progression. Below, we discuss the roles of the endoglin, TβRIII, neuropilin-1/2, and syndecan-2 in tumor initiation, progression and angiogenesis, as well as their influence on the tumor stroma.

3.2.1 Cancer Initiation and Progression

3.2.1.1 Endoglin

Germline mutations in the ENG gene have been reported in patients with early onset Juvenile polyposis (JP), a disease that is associated with the development of gastrointestinal malignancies (Sweet et al. 2005). However, a larger follow-up study failed to confirm this finding (Howe et al. 2007). While endoglin is primarily expressed on cells derived from the hemangioblast (i.e., endothelial and hematopoietic cells), there have been numerous reports of altered endoglin expression in cancer cells (see Table 3.2). While some studies suggest endoglin expression is increased in a variety of cancers, others have observed a decrease in endoglin expression. Collectively, these studies suggest that the role of endoglin in cancer initiation and progression may be context specific.

Endoglin has been reported to have complex roles in TGF-β-mediated processes, including cancer cell adhesion, proliferation, and migration (Conley et al. 2004; Sanz-Rodriguez et al. 2004; Lakshman et al. 2011; Romero et al. 2010). In many cancers, endoglin appears to act as a suppressor of cancer progression. Overexpression of endoglin in prostate cancer cells decreases proliferation, invasion, and metastasis in vivo by influencing TGF-β signaling (Lakshman et al. 2011). Similarly, overexpression of endoglin was reported to reduce the invasiveness and tumorigenicity of esophageal squamous cell carcinoma cells, although the effect of endoglin expression on TGF-β signaling was not examined (Wong et al. 2008). In a breast cancer metastasis model, endoglin overexpression was reported to block TGF-β-enhanced cell motility/invasion, resulting in reduced lung colonization (Henry et al. 2011). In contrast to these studies, endoglin expression has been reported to promote the anchorage-independent growth of Ewing sarcoma cells (Pardali et al. 2011).

How might endoglin influence cancer progression? Endoglin is able to regulate the adhesion and migration of many cell types, in some cases by influencing TGF-β signaling (Sanz-Rodriguez et al. 2004; Conley et al. 2004; Lee et al. 2008; Lee and Blobe 2007; Ray et al. 2010; Guerrero-Esteo et al. 1999). While different cell types utilize different mechanisms to regulate adhesion and migration, insights into the function of endoglin in cancer may be derived from studies on non-tumorigenic cells. The cytosolic domain of endoglin likely plays a crucial role in endoglin's functions, as it mediates interactions with several proteins. Endoglin binds zyxin and zyxin-related protein 1, which are cytoskeleton-interacting proteins that localize to focal adhesions (Conley et al. 2004; Sanz-Rodriguez et al. 2004). Therefore, endoglin may regulate the organization of the actin cytoskeleton and the adhesive properties of cancer cells by interacting with these two proteins. Like TβRIII, the

Table 3.2 Expression of TGF-β co-receptors in human cancers

Cancer type	Increased expression	Decreased expression	References
Bladder	Syndecan-2		Marzioni et al. (2009)
Breast	Neuropilin-1, Neuropilin-2, soluble Endoglin	TβRIII, Endoglin	Ghosh et al. (2008), Yasuoka et al. (2009), Staton et al. (2007), Dong et al. (2007), Hempel et al. (2007), Henry et al. (2011), Calabro et al. (2003), Davidson et al. (2010), Li et al. (2000), Takahashi et al. (2001)
Colon	Neuropilin-1, Neuropilin-2, TβRIII, soluble Endoglin, Syndecan-2	Neuropilin-1	Parikh et al. (2004), Grandclement et al. (2011), Gray et al. (2008), Gatza et al. (2012), Takahashi et al. (2001), Park et al. (2002), Ryu et al. (2009)
Endometrial	Endoglin	TβRIII	Florio et al. (2005)
Esophageal		Endoglin, Syndecan-2	Wong et al. (2008), Huang et al. (2009)
Ewing sarcoma	Endoglin		Pardali et al. (2011)
GI Tract	Neuropilin-1	Neuropilin-2	Hansel et al. (2004), Cohen et al. (2001)
Granulosa tumor		TβRIII	Bilandzic et al. (2009)
Kidney		TβRIII	Copland et al. (2003), Cooper et al. (2010)
Leukemia	Neuropilin-1, TβRIII, soluble Endoglin		Younan et al. (2012), Klein et al. (2001), Jelinek et al. (2003), Calabro et al. (2003)
Liver	Neuropilin-1	TβRIII	Berge et al. (2011), Bae et al. (2009)
Melanoma	Neuropilin-1, Neuropilin-2, Endoglin, Syndecan-2		Lacal et al. (2000), Rushing et al. (2012), Pardali et al. (2011), Lee et al. (2009)
Mesothelioma	Syndecan-2		Gulyas and Hjerpe (2003)
Multiple myeloma		TβRIII	Lambert et al. (2011)
Neuroblastoma		TβRIII	Iolascon et al. (2000)
Non-Hodgkin's lymphoma	TβRIII		Woszczyk et al. (2004)
Non-small cell lung	Neuropilin-1, Neuropilin-2, soluble Endoglin	TβRIII	Kawakami et al. (2002), Finger et al. (2008), Kopczynska et al. (2012)
Osteosarcoma	Neuropilin-2	Syndecan-2	Handa et al. (2000), Orosco et al. (2007)
Ovarian	Neuropilin-1, Neuropilin-2, soluble Endoglin	TβRIII	Drenberg et al. (2009), Baba et al. (2007), Bock et al. (2011), Hempel et al. (2008)

(continued)

Table 3.2 (continued)

Cancer type	Increased expression	Decreased expression	References
Pancreatic	Neuropilin-1, Neuropilin-2, Syndecan-2	TβRIII	Parikh et al. (2004), Cohen et al. (2002), Li et al. (2004), Gordon et al. (2008)
Prostate	Neuropilin-1, Neuropilin-2, Endoglin, soluble Endoglin, Syndecan-2	TβRIII, Endoglin	Latil et al. (2000), Yacoub et al. (2009), Turley et al. (2007), Lakshman et al. (2011), Kassouf et al. (2004), Shariat et al. (2008), Popovic et al. (2010)
Salivary adenoid cystic carcinoma	Neuropilin-2		Cai et al. (2010)
Squamous cell carcinoma		TβRIII, Endoglin	Wong et al. (2008), Meng et al. (2011)
Thyroid	Neuropilin-2		Finley et al. (2004)

cytosolic domain of endoglin can associate with GIPC and β-arrestin 2 (Lee et al. 2008; Lee and Blobe 2007; Chen et al. 2003). The interaction between endoglin and GIPC was recently shown to regulate TGF-β pathway signaling events, endothelial cell migration, and capillary tube formation via the recruitment of PI3K and Akt (Lee et al. 2012). β-arrestin 2, in contrast, mediates the internalization of endoglin, resulting in changes in Erk activation and localization as well as endothelial cell migration in a TGF-β-dependent manner (Lee and Blobe 2007). Whether similar pathways mediate endoglin function in cancer cells remains to be defined. In addition, the role of endoglin in mediating the effects of BMPs and Activin in the context of cancer progression has not been explored.

3.2.1.2 TβRIII

While TβRIII mutations have not been reported in human cancers, a recent study demonstrated that myxoinflammatory fibroblastic sarcomas and hemosiderotic fibrolipomatous tumors contained a recurrent chromosome t(1:10) rearrangement, which involves the TGFBR3 gene (Antonescu et al. 2011), suggesting a potential role for TβRIII in the pathogenesis of these diseases. Additionally, the progressive loss of TβRIII has been documented in multiple cancers (see Table 3.2).

Importantly, loss of TβRIII expression during cancer progression is associated with increasing cancer grade and stage and a poorer patient prognosis, supporting an important functional role for loss of TβRIII expression (Lambert et al. 2011; Dong et al. 2007; Meng et al. 2011; Turley et al. 2007). In many of these cases, the loss of TβRIII leads to disrupted TGF-β signaling. Loss of TβRIII expression in human cancers can occur by multiple mechanisms including loss of heterozygosity (Dong et al. 2007; Turley et al. 2007; Finger et al. 2008), gene silencing by promoter methylation (Turley et al. 2007; Cooper et al. 2010; Hempel et al. 2007), and the

negative regulation of TGFBR3 promoter by TGF-β or BMP ligands (Hempel et al. 2008; Gordon et al. 2009). As many cancers are associated with increased TGF-ligand production (Massague 2008), the downregulation of TβRIII expression by ligand is likely a common event. Indeed, the ability of TGF-β ligand to downregulate TβRIII expression has been documented in breast (Dong et al. 2007; Hempel et al. 2007), ovarian (Hempel et al. 2007), and non-small cell lung cancer cell lines (Finger et al. 2008).

Consistent with its frequent loss of expression in a broad spectrum of human cancers, TβRIII has been demonstrated to inhibit both ligand-dependent and independent cancer initiation and cancer progression in preclinical models of these cancers. siRNA-mediated silencing of TβRIII expression in normal murine mammary gland cells (NMuMG) was sufficient to induce tumorigenicity in mice (Criswell and Arteaga 2007). In a reciprocal manner, restoring TβRIII expression decreased tumorigenicity in breast (Sun and Chen 1997; Bandyopadhyay et al. 2002a; Bandyopadhyay et al. 1999a), prostate (Turley et al. 2007), non-small cell lung cancer (Finger et al. 2008), and renal cell carcinoma models (Copland et al. 2003).

Mechanistically, TβRIII has been reported to influence different aspects of tumor biology in a context-dependent manner. Thus, in the context of breast cancer (Sun and Chen 1997), renal cell carcinoma (Copland et al. 2003; Margulis et al. 2008), and multiple myeloma (Lambert et al. 2011), TβRIII inhibited proliferation by mediating TGF-β signaling, while in the context of renal cell carcinoma (Copland et al. 2003; Margulis et al. 2008), TβRIII stimulated apoptosis. Most consistently, in the context of multiple myeloma (Lambert et al. 2011) and cancers of the breast (Dong et al. 2007; Lee et al. 2010), pancreatic (Gordon et al. 2008), ovary (Hempel et al. 2008; Bilandzic et al. 2009) prostate (Turley et al. 2007), and lung (Finger et al. 2008), TβRIII inhibited cancer cell migration and invasion by attenuating TGF-β signaling.

TβRIII inhibits migration and invasion through multiple mechanisms. In some contexts, TβRIII inhibits migration/invasion through the generation of soluble TβRIII, which is thought to bind and sequester free TGF-β ligand in the extracellular space, thereby inhibiting its pro-migratory effects (Bandyopadhyay et al. 2002a; Bandyopadhyay et al. 2002b; Bandyopadhyay et al. 2005; Bandyopadhyay et al. 1999b; Dong et al. 2007; Gordon et al. 2008; Sun and Chen 1997; Finger et al. 2008). TβRIII also inhibits migration and invasion through soluble TβRIII-independent mechanisms, with the interaction between TβRIII and GIPC mediating the anti-migratory and anti-invasive effects of TβRIII in the context of breast cancer (Lee et al. 2010). Through its interaction with β-arrestin2, TβRIII can also negatively regulate the pro-migratory and pro-invasive transcription factor NFκB (Criswell and Arteaga 2007; You et al. 2009). Additionally, TβRIII inhibits the intrinsic ability of epithelial cells to migrate through its interaction with β-arrestin2 (Mythreye and Blobe 2009b), with TβRIII mediating β-arrestin2-dependent constitutive activation of Cdc42 and alterations in the actin cytoskeleton (Mythreye and Blobe 2009b). Interestingly, these effects are also independent of TβRIII's role in ligand presentation (Mythreye and Blobe 2009b). TβRIII also functions to inhibit TGF-β and BMP-mediated EMT and EMT-associated increases in migration and

invasion (Gordon et al. 2008; Gordon et al. 2009; Criswell and Arteaga 2007; Mythreye and Blobe 2009b; Hempel et al. 2007). Finally, TβRIII may influence cell migration and invasion by regulating cell adhesion. Indeed, TβRIII has been shown to influence the interaction between multiple myeloma cells and bone marrow stromal cells and to regulate the adhesive properties of ovarian cancer, breast cancer, and granulosa cell tumors (Lambert et al. 2011; Mythreye et al. 2012; Bilandzic et al. 2009). Mechanistically, TβRIII affects focal adhesion dynamics by regulating the localization of α5 integrin (Mythreye et al. 2012).

In contrast to most cancers, TβRIII expression is increased at the protein level in colon cancer, where TβRIII is associated with an inhibition of chemotherapy-induced apoptosis, increased TGF-β signaling, colony formation in soft agar, migration, and ligand-stimulated proliferation in vitro and tumorigenicity in vivo (Gatza et al. 2012). The reasons for the opposing effects of TβRIII on colon versus other cancers are currently unknown. However, as K-Ras is mutated in up to 50 % of colon cancer patients (Benhattar et al. 1993), crosstalk between K-Ras and the TGF-β pathway may be involved. Interestingly, increased TβRIII expression has also been observed in non-Hodgkin's lymphoma and B-cell chronic lymphocytic leukemia (Woszczyk et al. 2004; Klein et al. 2001; Jelinek et al. 2003), although the effect of TβRIII on the biology of these cancers is unknown

3.2.1.3 Neuropilin-1/2

As co-receptors for VEGF and semaphorin ligands, the neuropilins have important roles in regulating angiogenesis. However, the neuropilins are also expressed on cancer cells (see Table 3.2) and function as TGF-β co-receptors in regulating the TGF-β responsiveness of cancer cells (Staton et al. 2007; Mak et al. 2010; Grandclement et al. 2011). Like endoglin, neuropilin-1/2 has context-dependent effects in regulating cancer progression.

Mechanistically, neuropilin-1/2 may promote cancer progression by activating latent TGF-β and enhancing signaling. This has been shown to occur in regulatory T-cells as well as breast cancer cells (Glinka and Prud'homme 2008). Furthermore, VEGF and neuropilin-1 have been shown to directly promote epithelial-mesenchymal transition (EMT) in prostate cancer cells (Mak et al. 2010), and the knockdown of neuropilin-2 in colon cancer cells has also been associated with EMT (Grandclement et al. 2011). As EMT can also be triggered by TGF-β, the neuropilins may also aid in TGF-β-mediated EMT, although this has not yet been directly demonstrated

3.2.1.4 Syndecan-2

While syndecan-2 does bind to TGF-β, it is currently unknown what roles this binding may play in cancer development and progression. However, altered syndecan-2 expression has been observed in several cancers (see Table 3.2), and syndecan-2 has

been shown to positively regulate cancer cell migration and invasion in a variety of cancer types (Choi et al. 2010; Lee et al. 2009; Orosco et al. 2007; Ryu et al. 2009).

Syndecan-2's role in colon cancer has been most thoroughly investigated. Increased mRNA and protein expression of syndecan-2 have been observed in human colon adenocarcinoma tissue samples in comparison with neighboring normal epithelium (Ryu et al. 2009). Additionally, high expression of syndecan-2 has been observed in colon cancer cell lines. This increased expression is associated with increased proliferation, migration, and invasion (Ryu et al. 2009; Park et al. 2002; Choi et al. 2010). Syndecan-2 regulates colon and fibrosarcoma cancer cell migration and invasion through Tiam1-dependent Rac activation (Park et al. 2005; Choi et al. 2010). Additionally, MMP7 activity and integrin α2 are upregulated upon syndecan-2 expression in colon cancer cells, which may influence adhesion, migration, and invasion (Ryu et al. 2009; Choi et al. 2010).

3.2.2 Cancer Stem Cells

In some cancers, a small percentage of tumor cells have been isolated that possess the unique capability of sustained self-renewal and differentiation (Grotenhuis et al. 2012). As such features are common to stem cells, these cells are referred to as cancer stem cells (CSCs) or tumor-initiating cells. CSCs are unique in that, unlike the majority of tumor cells, they are typically more chemoresistant and are more effective in generating xenografts in mouse models (Grotenhuis et al. 2012). CSCs are also capable of giving rise to non-CSC populations and thus can recapitulate the heterogeneity of the tumor from which they were isolated. Therefore, it has been suggested that CSCs can drive tumor initiation or recurrence. For a recent review on CSCs, please see Grotenhuis et al. 2012.

While endoglin, TβRIII, and the neuropilins have been shown to have roles primarily in TGF-β-mediated angiogenesis and/or cancer cell migration and invasion, a few reports have identified these co-receptors as CSC markers. In a cohort of matched primary/recurrent ovarian cancer specimens, along with the expression of several known markers of ovarian CSCs, endoglin was found to be significantly increased in persistent tumors compared to matched primary tumors (Steg et al. 2012). Additionally, endoglin-positive cells isolated from a rhabdoid meningioma surgical sample exhibited increased proliferation and had an increased ability to form single-cell tumor spheres in vitro compared to cells lacking endoglin expression (Hu et al. 2012). Interestingly, the endoglin-positive cells were found to have several cell surface markers in common with mesenchymal progenitor cells, which are multipotent and are the precursor cell type for several tissues including bone, muscle, and fat (Hu et al. 2012; Houthuijzen et al. 2012).

With regard to TβRIII, a lack of TβRIII expression has been associated with prostate CSCs. Sharifi et al. showed that prostate cancer cells lacking TβRIII expression had higher levels of the prostate CSC marker CD133 than tumor cells that were positive for TβRIII expression, suggesting that the loss of TβRIII may increase the population of stem-like cells (Sharifi et al. 2007).

Notably, skin squamous cell carcinoma CSCs were found to lack endoglin expression; however, these cells did express high levels of neuropilin-1 (Beck et al. 2011). The expression of neuropilin-1 was shown to be critical for the ability of these CSCs to promote VEGF-stimulated tumor growth in a mouse model (Beck et al. 2011). Hamerlik et al. also detected neuropilin-1 expression on CSCs isolated from glioblastoma multiforme tumors (Hamerlik et al. 2012). Here, neuropilin-1 was found to stabilize VEGFR2 expression, thereby enhancing the self-renewal capacity and viability of the CSCs (Hamerlik et al. 2012). While it is unknown what role TGF-β family ligands play in CSCs, the expression of these TGF-β co-receptors on CSCs provides one more mechanism by which co-receptors can regulate the pathogenesis of human cancers.

3.2.3 Cancer-Associated Angiogenesis

3.2.3.1 Endoglin

Endoglin is intricately involved in the process of angiogenesis, which is required to sustain primary tumor growth and metastasis (Folkman et al. 1989; Neri and Bicknell 2005; Molema and Griffioen 1998). Endoglin expression is higher in nascent blood vessels, where it controls cell proliferation, migration, and capillary tube formation (Li et al. 1999; Fansatti et al. 2001; Lebrin et al. 2004; Goumans et al. 2002; Ma et al. 2000; Cheifetz et al. 1992; Li et al. 2000; Bourdeau et al. 1999; McAllister et al. 1994). While endoglin is highly expressed during angiogenesis in multiple cancer types, its expression is typically absent on vessels in normal tissues (Fansatti et al. 2001). Because of its lack of expression in normal vasculature, endoglin has been extensively used as a marker for measuring microvessel density (MVD), which is inversely correlated with prognosis (Fansatti et al. 2001; Brewer et al. 2000; Saad et al. 2004; Wikstrom et al. 2002; Kumar et al. 1999; Shariat et al. 2008; Schimming and Marme 2001; Tanaka et al. 2001). Endoglin has emerged as a powerful marker of tumor-associated neovascularization, with anti-endoglin antibodies being investigated to visualize tumors and their metastases (Fonsatti et al. 2010).

The dependence of tumors on the pro-angiogenic functions of endoglin has also made it an attractive target for anti-tumor therapies (Dallas et al. 2008). Several in vivo studies have revealed endoglin to be an important therapeutic target. First, endoglin haploinsufficient mice were shown to have decreased lung tumor vascularization and growth compared to their endoglin +/+ littermates (Duwel et al. 2007). This may be due to increased endothelial cell apoptosis, as endoglin was also shown to regulate eNOS levels and to prevent apoptosis in hypoxic endothelial cells (Duwel et al. 2007). Additionally, the suppression of tumor growth and metastasis and an increased survival rate have been observed using anti-endoglin antibodies alone or antibodies conjugated to immunotoxins or radiolabels in mice (Seon et al. 1997; Matsuno et al. 1999; Tabata et al. 1999; Tsujie et al. 2008; Takahashi et al. 2001;

Uneda et al. 2009). A phase I clinical trial using an anti-endoglin antibody (TRC105) in patients with advanced refractory cancers was recently reported, with the treatment is well tolerated, and resulting in disease control in nearly half of the patients (Rosen et al. 2008; Rosen et al. 2012) (NCT00582985).

3.2.3.2 TβRIII

Like endoglin, a few studies have implicated TβRIII in tumor-associated angiogenesis. In a xenograft mouse model of breast cancer, decreased tumor-associated angiogenesis was observed in mice injected with TβRIII-expressing cells vs. control cells (Dong et al. 2007). The administration of recombinant soluble TβRIII also decreased tumor vascularization in xenograft models of breast and prostate cancer (Bandyopadhyay et al. 2005; Bandyopadhyay et al. 2002a), and soluble TβRIII has been shown to inhibit the ability of human endothelial cells to form tubes in an in vitro Matrigel assay (Bandyopadhyay et al. 2002b). In contrast to these reports, SERPINE1, which is a known inhibitor of angiogenesis, was found to be downregulated in a gene array performed on prostate cancer cells lacking TβRIII expression (Sharifi et al. 2007). Taken together, these studies suggest an anti-angiogenic function for TβRIII.

3.2.3.3 The Neuropilins

Most of the work regarding neuropilin-1/2 and tumor angiogenesis has focused on their role as VEGF co-receptors, and little attention has been given to their potential role in angiogenesis as TGF-β co-receptors. The neuropilins are, however, clearly involved in tumor-associated angiogenesis. In breast cancer specimens, increased neuropilin-1 expression was observed in both smooth muscle and endothelial cells within the areas of invasion (Stephenson et al. 2002). Additionally, non-small cell lung cancer patients whose tumors expressed both neuropilins had increased vessel counts compared to those patients that lacked co-expression (Kawakami et al. 2002). The neuropilin ligands Sema3A and Sema3F (class 3 semaphorins) may also regulate tumor angiogenesis as they have been shown to inhibit the pro-angiogenic effects of VEGF in endothelial cells (Staton et al. 2007; Wild et al. 2012). Both of these ligands were found to inhibit endothelial cell migration, proliferation, and tube formation (Narazaki and Tosato 2006; Serini et al. 2003). As the interaction between neuropilin-1 and VEGF is important for tumor angiogenesis, several current strategies for anti-tumor therapy revolve around the inhibition of neuropilin-1, VEGF, both, or their interaction (Geretti and Klagsbrun 2007; Pan et al. 2007; Jia et al. 2010; Jia et al. 2006; Soker et al. 1997; von Wronski et al. 2006). The role of the neuropilins in angiogenesis as it relates to their role as TGF-β co-receptors requires additional investigation.

3.2.3.4 Syndecan-2

Very little is known regarding syndecan-2's role in tumor-associated angiogenesis. However, high syndecan-2 expression has been reported in the microvasculature of mouse gliomas (Fears et al. 2006), while downregulation of syndecan-2 resulted in decreased endothelial cell motility and a reduced formation of capillary tube-like structures (Fears et al. 2006). The ability of syndecan-2 to promote angiogenesis was found to be due to increased levels of soluble syndecan-2, which were increased during glioma progression (Fears et al. 2006).

3.2.4 TGF-β Co-receptors and Stromal Components

3.2.4.1 Endoglin

Endoglin is weakly expressed in some stromal cell types (Rokhlin et al. 1995; Gougos et al. 1992). In a mouse model of prostate cancer progression, endoglin (+/−)-derived tumors lacked the typically observed infiltration of cancer-associated fibroblasts (Romero et al. 2011). Therefore, endoglin may act to support the viability of CAFs in the tumor microenvironment by promoting neovascularization and growth. In a co-culture model of the prostate cancer cell line C4-2B and the bone marrow stromal cell line HS-27a, endoglin was significantly downregulated in the stromal cells only upon co-culture (O'Connor et al. 2007). This loss of endoglin resulted in attenuated TGF-β signaling and decreased stromal cell proliferation (O'Connor et al. 2007). Thus the prostate cancer cells, upon interaction with bone marrow stromal cells, may alter stromal cell TGF-β signaling to promote the formation of bone metastases (O'Connor et al. 2007).

3.2.4.2 TβRIII

Similar to cancer cells, the expression of TβRIII mRNA is significantly reduced in CAFs in oral squamous cell carcinoma (Meng et al. 2011). As increased amounts of TGF-β ligand are present within the oral cancer microenvironment, ligand-induced downregulation of TβRIII expression was thought to be a potential mechanism for downregulation (Meng et al. 2011). TβRIII also has a potential role in exosomes, which are secreted vesicles that participate in intercellular communication (Webber et al. 2010). Exosomes can deliver protein, mRNA, or microRNAs to recipient cells. Interestingly, TGF-β was recently shown to be expressed by prostate and breast cancer-derived exosomes (Webber et al. 2010). This exosomal TGF-β was capable of promoting fibroblast–myofibroblast differentiation (Webber et al. 2010). The association of TGF-β with the exosomal membrane was linked with TβRIII expression, suggesting that TβRIII acts as a TGF-β anchor on cancer-associated exosomes (Webber et al. 2010).

3.2.4.3 The Neuropilins

Neuropilin-1 has been shown to promote the activation of fibroblasts to myofibroblasts, which are important regulators of cancer progression (De Wever and Mareel 2003). Decreasing neuropilin-1 expression in fibroblasts enhanced Smad1/5 signaling but reduced Smad2/3 signaling (Cao et al. 2010b). Mechanistically, an interaction between neuropilin-1 and endogenous TβRII was associated with decreased fibroblast proliferation and a reversion to a quiescent state (Cao et al. 2010b). In the immune system, neuropilin-1 is expressed by regulatory T cells and has been shown to activate latent TGF-β in this cell type (Glinka and Prud'homme 2008). Indeed, T cells expressing neuropilin-1 could efficiently bind latent TGF-β1. However, this was not observed in cells lacking neuropilin-1 (Glinka and Prud'homme 2008). Similar results were observed in a breast cancer cell line, suggesting that this role of neuropilin-1 is common and may be important in cancer progression (Glinka and Prud'homme 2008).

3.3 TGF-β Co-receptors in Vascular Disease

As TGF-β plays a key role in physiological angiogenesis, it is not surprising that animal models and human studies have implicated TGF-β superfamily signaling in the initiation and progression of many vascular disorders. Due to the role of the co-receptors in regulating the TGF-β pathway, direct mutations or disruptions in their function have been tied to these disorders as well.

3.3.1 Endoglin

A critical role for endoglin in angiogenesis has come from studies of mice lacking this co-receptor. Endoglin-deficient mice die at midgestation (day 10.5) of angiogenic and cardiovascular defects (Sorensen et al. 2003). During development, endoglin-deficient mice lose structural, molecular, and functional distinctions between arteries and veins (Li et al. 1999). Additionally, these mice fail to form mature blood vessels in the yolk sac (Li et al. 1999). Such studies indicate that altered TGF-β signaling is the cause of the phenotypes observed in endoglin null mice and also establish a functional link between endoglin and the TGF-β pathway. Vasculogenesis in the endoglin null mice is normal, but angiogenesis and remodeling of the primary vascular plexus are impaired. The mice exhibit poor vascular smooth muscle cell (VSMC) development that results in dilation and rupture of the vascular channels. In the heterozygous mice, disease severity increases with age and can include the rupture of major vessels.

Mutations in endoglin can also lead to the vascular disease hereditary hemorrhagic telangiectasia (HHT), or Rendu–Osler–Weber syndrome. HHT is a rare autosomal dominant disorder that is characterized by arteriovenous malformations or localized abnormal arteriovenous connections that affect both the microvasculature and large vessels. HHT patients typically manifest spontaneous epistaxis (nose bleeds), cutaneous telangiectases (small dilated blood vessels), and arteriovenous malformations in internal organs. Additional features include highly variable expression and variable, age-dependent penetrance. More than 150 mutations have been reported in endoglin including deletions, insertions, missense mutations, and splice site changes, all of which result in reduced levels of endoglin on the surface of endothelial cells (McAllister et al. 1994). Patients with HHT also have significantly lower amounts of endoglin in peripheral monocytes compared to normal individuals (Abdalla and Letarte 2006). Interestingly, most of the known mutations in endoglin are within exons 1–12, which encode the extracellular domain, and very few mutations have been observed in exons 13 and 14, which encode the transmembrane and cytoplasmic domains. Many of these alterations result in deficient angiogenesis, i.e. the sprouting of new vessels from preexisting ones. One proposed explanation for the development of HHT is that mutations in endoglin result in misfolded proteins that are either incapable of heretodimerizing or are unable to reach the cell surface, both of which would account for the halpoinsufficiency that is observed in HHT1.

Preeclampsia, a systemic, pregnancy-associated hypertensive disorder, has also been linked with endoglin. Preeclampsia appears to originate in the placenta and is characterized by endothelial cell dysfunction. As such, several authors have reported changes in the expression of placental anti-angiogenic factors in preeclamptic patients [reviewed in (Maynard and Karumanchi 2011)]. Soluble endoglin has been found to be elevated in preeclampsia and has been proposed as a viable marker for this disorder as well. However, the molecular basis for preeclampsia and the exact role of soluble endoglin in this disorder remain unknown.

3.3.2 The Neuropilins

Neuropilin-1 mediates normal developmental angiogenesis, which has been observed in mouse and zebrafish models. Neuropilin-1 has also been shown to mediate pathological angiogenesis in tumors and retinal disease, particularly in the context of VEGF signaling, wherein neuropilin 1, which is highly expressed in the retinal endothelial cells, is upregulated by both VEGF and hypoxia to regulate a positive feedback mechanism in retinal neovascularization. As such, most of neuropilin-1/2 functions in angiogenesis and vascular biology are related to its roles as a receptor for VEGF and as a co-receptor for VEGFR2 (Soker et al. 1997). Currently, much less is known about the ability of neuropilin-1 to regulate TGF-β's function in angiogenesis.

3.3.3 Syndecan-2

Syndecan-2 has been shown to regulate endothelial cell functions in vitro, including migration and cytoskeletal organization (Noguer et al. 2009). These functions may in turn contribute to its roles in capillary tube formation. Studies in zebrafish have demonstrated specific roles for syndecan-2 in regulating sprouting during angiogenesis (Chen et al. 2004a). Notably, the role of syndecan-2 in regulating TGF-β signaling in the context of endothelial biology has not been established and requires further exploration.

3.4 TGF-β Co-receptors and Developmental Defects

3.4.1 Endoglin

Genes that are required for proper vascular development also often affect blood flow. In addition, genes that are expressed in the endocardial lining of the heart can affect cardiac development, as in the case for the TGF-β co-receptors including endoglin, TβRIII, neuropilin-1, and syndecan-2. In addition to endoglin's well-studied role in vascular development, endoglin knockout mice have revealed a role for endoglin in cardiovascular development. In endoglin null mice, the atrioventricular canal endocardium fails to undergo mesenchymal transformation and does not form cushions (Li et al. 1999). These heart valve formation defects may be due to a perturbation in TGF-β signaling. Interestingly, endoglin has also been detected in hematopoietic stem cells in adult bone marrow and in the AGM (aorta–gonal mesonephros) at E11.5 (Chen et al. 2002) as well as in fetal liver cells (Pimanda et al. 2008). In addition to cardiovascular defects, endoglin null mouse embryos also display anemia of the yolk sac, which was, until recently, assumed to be an indirect result of insufficient blood flow. However, recent evidence demonstrates that endoglin plays a direct role in hematopoiesis. Endoglin is expressed in the first hematopoietic progenitor cells during embryonic development where it has been shown to regulate TGF-β/BMP signaling during the initiation of hematopoiesis (Borges et al. 2012).

3.4.2 TβRIII

Similar to endoglin, the deletion of TβRIII in mice results in embryonic lethality due to failed coronary vessel development and palate fusion (Compton et al. 2007). TβRIII null mice exhibit significantly impaired coronary vasculogenesis, defects in the epicardium, and dysmorphic and distended vessels. Collectively, these phenotypes demonstrate a requirement for TβRIII during coronary vessel development,

which is essential for embryonic viability (Compton et al. 2007). More recently, an analysis of E13.5 TβRIII null embryos revealed a lower rate of epicardial cell proliferation and decreased epicardial-derived cell invasion. This effect is mediated by the interaction of TβRIII's cytoplasmic domain with GIPC (Sanchez et al. 2011), with loss of responsiveness to TGF-β and FGF2, two important regulators of epicardial cell behavior (Sanchez et al. 2011). In addition to its role in proper heart development, TβRIII has also been shown to participate in cord formation, fetal Leydig cell development, and the establishment of fetal endocrine testis function. Collectively, the observations implicate TGF-β superfamily members as regulators of early fetal testis structure and function (Sarraj et al. 2010). TβRIII mRNA has a distinct expression pattern during gonadogenesis, where it is expressed at higher levels in developing testes compared to developing ovaries. TβRIII is predominantly expressed by Leydig cells within the fetal testis interstitium; however, at birth, expression is shifted to the seminiferous cords, suggesting possible roles for TβRIII in the gonadogenesis (Sarraj et al. 2007). Interestingly, TβRIII can bind inhibin and can regulate ovarian and granulosa carcinogenesis through this interaction.

3.4.3 Neuropilin-1

Neuropilin-1 plays key roles in vascular biology. Neuropilin-1 mutant mouse embryos have impaired arterial differentiation, which appears to be independent of blood flow patterns (Jones et al. 2008). Neuropilin-1's role in arterial specification during vascular development is primarily due to its ability to regulate VEGF/VEGFR2 function. The function of neuropilin-1 in development can be additionally regulated at the transcriptional level. As such, the Notch signaling pathway, which directs cell fate decisions during embryonic development, has been shown to regulate the neuropilin-1 promoter to alter cell responsiveness to several ligands including VEGF (Sorensen et al. 2009) and, potentially, TGF-β.

In addition to its roles in vascular development (see angiogenesis), neuropilin-1 plays pivotal roles in the development of the neuronal system as a receptor for members of the class-3 semaphorin family of axonal guidance factors [reviewed in (Geretti and Klagsbrun 2007)]. Much less is known about its role in regulating TGF-β's role in developmental processes.

3.4.4 Syndecan-2

The role of syndecan-2 in development has been best studied in Xenopus embryos. Here, syndecan-2 has been shown to be a regulator of left–right development in the embryonic mesoderm (Kramer and Yost 2002). This function of syndecan-2 appears to be mediated via the functional interactions of its heparan sulfate glycosaminoglycan chains with the TGF-β superfamily cell–cell signaling molecule Vg1, which is

known to regulate early left–right development. Syndecan-2 is expressed in the ectoderm and likely presents Vg1 to the migrating mesoderm in a unilateral manner, demonstrating that syndecan-2 can function in cell signaling by directly affecting neighboring cells or in a cell-autonomous manner. Additional mechanistic studies indicate that PKCγ can phosphorylate syndecan-2 and that this phosphorylation event is required in the right-side ectodermal cells (Kramer et al. 2002). While studies in zebrafish have revealed a similar role for syndecan-2 in left–right development, knockdown experiments have also demonstrated a requirement for syndecan-2 in embryonic angiogenesis. During both zebrafish and mouse development, syndecan-2 is expressed in the mesenchymal cell layer surrounding the axial blood vessels, suggesting a potential role for this molecule in coordinating and organizing vascular development (Chen et al. 2004a). Syndecan 2 and VEGF are thought to act synergistically to facilitate angiogenesis.

3.5 TGF-β Co-receptors in Fibrosis

Fibrosis, which is characterized by the fibroblast-mediated deposition of the ECM proteins collagen and fibronectin, can play roles in diverse pathological conditions including the autoimmune disease systemic sclerosis (SSc), wound healing (particularly after myocardial infarction), Crohn's disease, pulmonary hypertension, and diabetic nephropathy. Hepatic fibrosis can also set the stage for the development of cirrhosis and, in some cases, hepatocellular carcinoma (HCC). TGF-β is a key regulator of the fibrotic processes [reviewed in (Hawinkels and Ten Dijke 2011)]. Not all of the TGF-β co-receptors have established roles in each of the pathological conditions associated with fibrosis; however, some common themes exist regarding the ability of the co-receptors to modulate TGF-β signaling during the fibrotic process.

3.5.1 Endoglin

Endoglin has been shown to be a negative regulator of TGF-β1 signaling in the intestinal fibroblast, where it modulates Smad3 phosphorylation, Smad binding element (SBE) promoter activity, connective tissue growth factor (CTGF) production, and collagen contraction (Burke et al. 2009). Endoglin is also upregulated in patients suffering from fibrosis or scleroderma, where increased soluble endoglin levels are present as well (Wipff et al. 2008; Coral-Alvarado et al. 2010; Dharmapatni et al. 2001). Endoglin can both stimulate and inhibit TGF-β-induced fibrosis, depending on the context. For instance, in human and rat cell line models using myoblasts and myofibroblasts, endoglin was shown to reduce cell responsiveness to TGF-β, thereby reducing collagen production via an ERK-dependent mechanism (Rodriguez-Barbero et al. 2006). In addition, fibroblasts derived from patients with fibrosis or SSc have higher basal levels of CTGF and collagen relative to healthy individuals, and the overexpression of endoglin in control fibroblasts (from healthy

donors) was shown to block TGF-β-induced CTGF expression. Collectively, these studies suggest an inhibitory role for endoglin in fibrosis (Holmes et al. 2011). In constrast, endoglin has also been shown to positively regulate fibrosis, especially in the context of cardiac fibroblasts. Here, endoglin expression is upregulated by TGF-β and angiotensin II, and blocking this upregulation resulted in reduced collagen II expression (Shyu et al. 2010), reviewed in (Maring et al. 2012). Fibroblasts from patients suffering from Crohn's disease have also been shown to express high levels of endoglin. Future studies on how endoglin expression is regulated should shed more light on the dichotomous roles of this co-receptor in fibrosis.

3.5.2 TβRIII

Given the structural and functional similarities between endoglin and TβRIII, similar roles for these two co-receptors in fibrosis could be predicted. Consistent with this idea, soluble TβRIII has been shown to inhibit fibrous airway obliteration (Liu et al. 2002) and can prevent myocardial fibrosis in spontaneously hypertensive rats (Hermida et al. 2009) via the sequestration of TGF-β. Like endoglin, TβRIII levels are elevated in patients with SSc, where TGF-β activity is enhanced (Holmes et al. 2011). Some evidence indicates that TβRIII expression is lost during fibroblast differentiation, suggesting that TβRIII may be required for suppressing lung fibrosis; however, the precise mechanism behind TβRIII's effects on fibroblastic differentiation remains to be elucidated (Ahn et al. 2010).

3.5.3 Neuropilin-1/2

Neuropilin-1 has been implicated as a regulator of the fibrotic response, as it has been suggested to amplify TGF-β and PDGF signaling in hepatic stellate cells (HSC) and in HCC. In two rat models of liver fibrosis, neuropilin-1 was found to be upregulated in activated HSCs, which exhibit the highly motile myofibroblast phenotype (Cao et al. 2010a). Neuropilin-1 overexpression has also been shown to increase cell motility and TGF-β-dependent collagen production (Cao et al. 2010a). While these studies reveal a role for neuropilin-1 in liver fibrosis as a modulator of multiple growth factor targets, additional in vivo fibrosis studies must be performed to clarify the exact effects of neuropilin-1 on TGF-β, PDGF, and VEGF signaling during this process.

3.5.4 Syndecan-2

Syndecan-2 levels are elevated in renal interstitial cells under diabetic conditions. In addition, syndecan-2 has been shown to modulate TGF-β's ability to increase matrix deposition in several cell lines, possibly via effecting TβRIII expression levels (Chen et al. 2004b). Whether syndecan-2's role in fibrosis is mediated via TβRIII or via a direct modulation of TGF-β signaling remains to be determined.

3.6 Conclusions and Perspectives

The TGF-β signaling pathway has essential roles in many physiological processes including development, growth, differentiation, cell cycle regulation, cytokine and ECM production. Deregulation of these events results in a broad spectrum of human disease. The TGF-β co-receptors have diverse roles in regulating and mediating the TGF-β response. They may perform their actions through the regulation of signaling either in a cell autonomous or a non-cell autonomous manner through regulated ectodomain shedding, by localizing signaling in a spatio-temporal manner, or by orchestrating signaling with other growth factor receptors. Through a combination of these functions, the TGF-β co-receptors play essential roles in regulating physiological and pathophysiological processes in a tissue- and context-dependent manner. While the current body of literature supports complex roles for the co-receptors in mediating TGF-β's effects, many questions remain in terms of how these receptors function under physiological circumstances and during disease progression. Additional complexity exists due to the ability of the TGF-β co-receptors to bind multiple ligands through their extracellular domains as well as other proteins through their cytoplasmic domains. Defining the precise nature and relationship of these interactions and their functional consequences remains a challenge, particularly in determining which interactions mediate the biological functions of individual co-receptors. Further refining the structural domains mediating these interactions and assessing the role of loss of function mutants in vitro and in vivo should provide further insight into TGF-β co-receptor function. Despite these challenges, additional studies regarding the roles and mechanisms of action of the TGF-β co-receptors in signaling, biology, and human disease should advance our ability to target the TGF-β signaling pathway and these co-receptors in human disease.

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Part II
Cancer, Fibrosis, and Inflammation

Chapter 4
TGF-β in Cancer Stem Cells

Hiroaki Ikushima and Kohei Miyazono

Abstract Cancer stem cells are tumor cells characterized by their ability to induce tumorigenesis and to self-renew. Recent research advance in cancer research field has demonstrated heterogeneity in cancer cell population and established the cancer stem cell model. Simultaneously, this model has shed new light on the mechanism of cancer recurrence, tumor metastasis, and several other cancer-related phenomena. The most important issue of cancer stem cells in the clinical situation is their resistibility against conventional therapies. The mechanisms in which cancer stem cells maintain their stem-like abilities have thus been energetically studied in the last decade. TGF-β is a well-recognized factor responsible for maintenance of normal tissue stem cells. In addition to that, recent studies have unveiled the roles of TGF-β signaling in cancer stem cells. In this chapter, we first consider the basic concept of the cancer stem cell model and describe their special characters in cancer cell population. We then discuss the roles of TGF-β signaling in cancer stem cells, focusing on some types of cancers. We also address perspectives on TGF-β signaling as a potential target against cancer stem cells.

Keywords Cancer stem cells • Heterogeneity • Niche • SP cells • Tumorigenicity

Abbreviations

ABC	ATP-binding cassette
ALDH	Aldehyde dehydrogenase
AML	Acute myeloid leukemia
CML	Chronic myelogenous leukemia

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EMT	Epithelial–mesenchymal transition
FOXO	Forkhead O transcription factor
LIF	Leukemia inhibitory factor
MP	Main population
ROS	Reactive oxygen species
SP	Side population

4.1 Cancer Stem Cell Model

The basic principle of the cancer stem cell model is that not all cells in tumors are equal. In the cancer stem cell model, like the growth of normal proliferative tissues such as skin, intestinal epithelium, and bone marrow, the growth of tumors is accelerated by limited numbers of cells that have capacity to self-renew and have the exclusive ability to drive tumor progression (Reya et al. 2001; Jordan et al. 2006; Lobo et al. 2007; Visvader and Lindeman 2008). The bulk of a tumor consists of rapidly proliferating cells and differentiated non-dividing cells. A defined subset of cancer cells, or cancer stem cells, can give rise to more differentiated cancer cells (Fig. 4.1).

Two important observations led to the hypothesis that cancer stem cells may be responsible for tumor initiation and progression. One is that not all cells within a tumor are identical. Although most tumors arise from a single transformed cell, there are many different types of cells in a tumor. This observation is known as tumor heterogeneity (Park et al. 1971). The other observation is that a certain number of cancer cells were required to grow a tumor in a recipient mouse (Hamburger and Salmon 1977). In contrast, in the model in which cancer cells are usually clonal in origin and every cancer cell has the potential to form a new tumor (clonal evolution model), even a few cancer cells would be able to form new tumors.

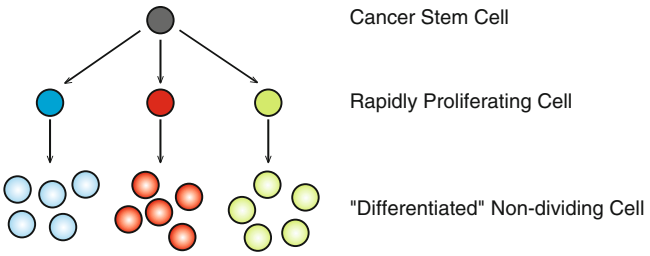

Fig. 4.1 In the cancer stem cell model, only a rare subpopulation of cells within the tumor, the cancer stem cells, has the capacity to generate and maintain a tumor. They can give rise to more differentiated cancer cells, which in turn form a hierarchy

4.2 History of Cancer Stem Cell Model

The concept of a cancer stem cell is not new and the connection between tumors and stem cells has a long history (Clevers 2011; Nguyen et al. 2012). In 1937, Furth and Kahn reported that a single cell from mouse leukemia could initiate a new tumor in a recipient mouse and that the tumor in a recipient mouse shares pathological characters similar to those of the original tumor (Furth and Kahn 1937). In 1941, Jackson and Brues showed that malignant teratocarcinomas contain many types of differentiated cells (Jackson and Brues 1941), suggesting their origin from tumorigenic cells with multilineage potential. Furthermore, in 1963, Bruce and Van Der Gaag reported the first in vivo colony assay for a mouse lymphoma-initiating cell (Bruce and Van Der Gaag 1963). In 1960s, studies by Pierce and his colleagues demonstrated that the morphologically undifferentiated cells in teratocarcinomas showed high tumorigenic activities and could differentiate into multiple differentiated non-tumorigenic cell types (Kleinsmith and Pierce 1964). These studies implicated these undifferentiated cells as candidate teratocarcinoma stem cells. Pierce's group also showed that, in mouse squamous cell carcinoma, well-differentiated cancer cells were derived from undifferentiated cells, using radiolabeling techniques (Pierce and Wallace 1971). From these and other experiments, Pierce defined the cancer stem cell concept as "a concept of neoplasms, based upon developmental and oncological principles, states that carcinomas are caricatures of tissue renewal, in that they are composed of a mixture of malignant stem cells, which have a marked capacity for proliferation and a limited capacity for differentiation under normal homeostatic conditions, and of the differentiated, possibly benign, progeny of these malignant cells" (Pierce and Speers 1988).

In 1970s, epoch-making discoveries were reported in the cancer research field: mutations in proto-oncogenes and tumor suppressor genes were found to cause most human cancers. After the existence of proto-oncogenes and tumor suppressor genes was established, the focus of cancer research shifted to the clonal evolution model as symbolized by the Knudson's two-hit theory and the Vogelstein's multistep carcinogenesis model. In the clonal evolution model, "tumor progression results from acquired genetic variability within the original clone allowing sequential selection of more aggressive sublines" (Nowell 1976; Merlo et al. 2006).

Until 1990s, it had been virtually impossible to directly prove the existence of cancer stem cells in human malignant tumors. However, development of immunocompromised mice, progression of cell sorting techniques, accumulation of knowledge on normal stem cells, and a variety of other scientific progression led Dick and his colleagues to revive the study of functional heterogeneity in leukemias (Lapidot et al. 1994; Bonnet and Dick 1997). They reported that only a subset of acute myeloid leukemia (AML) cells were able to transplant AML into immunodeficient recipient mice. These tumorigenic cells were defined as $CD34^{+}CD38^{-}$, suggesting similarities between leukemia stem cells and normal hematopoietic stem cells. In addition, the xenograft assay allowed measurement of the frequency of the leukemia stem cells; the order of one per million tumor cells. The remaining AML cells that

grew from the transplanted cells were in various stages of differentiation. These data suggest that heterogeneity in leukemias reflects the similar organizational hierarchy to that in normal blood cells.

Clarke and his colleagues applied these concepts and approaches to breast cancer cells and reported that only a minor fraction of breast cancer cells can form a tumor (Al-Hajj et al. 2003). This report was the first reported isolation and characterization of cancer stem cells from solid tumors. They isolated breast cancer stem cells on the basis of surface marker expression ($CD44^+CD24^-$) and tumor regeneration potential in the mammary fat pads of NOD/SCID mice. The tumorigenic subpopulation could be serially passaged and, even after secondary and tertiary transplants, the new tumors showed histopathological characteristics similar to the original tumor. There were no significant differences in cell morphology between the tumorigenic and non-tumorigenic breast cancer cells. This study was followed by similar studies on other solid tumors such as brain tumors (Singh et al. 2003; Singh et al. 2004; Galli et al. 2004), melanomas (Fang et al. 2005; Schatton et al. 2008), prostate cancers (Patrawala et al. 2006; Patrawala et al. 2007; Li et al. 2008), ovarian cancers (Zhang et al. 2008; Curley et al. 2009; Stewart et al. 2011), and colon cancers (O'Brien et al. 2007; Ricci-Vitiani et al. 2007; Dalerba et al. 2007a). In each type of cancer stem cells, small numbers of cells defined by specific markers have ability to form tumors in immunodeficient mice and the transplanted tumors recapitulate the heterogeneity of the original tumors. However, the quantification of cancer stem cells in a population of tumor cells is never absolute, but instead depends on the tumor-initiating assay used to test for their presence (Quintana et al. 2008). Furthermore, recent studies have suggested that reversible regulatory mechanisms allow cancer cells to dynamically cycle between a tumor-initiating "cancer stem cell" state and a non-tumor-initiating cell state (Sharma et al. 2010; Roesch et al. 2010; Quintana et al. 2010; Scheel and Weinberg 2011).

Thus, the clonal evolution model and the cancer stem cell model constitute two major frameworks for interpreting clinical and experimental phenomena of malignant tumors. Of course, these two models are not mutually exclusive, as evidenced by substantial genetic heterogeneity in populations of putative cancer stem cells (Anderson et al. 2011; Notta et al. 2011).

4.3 Characteristics of Cancer Stem Cells

4.3.1 Tumorigenic Activity and Self-Renewal Capacity

The most characteristic feature of cancer stem cells is their high tumorigenic activity. This character is usually proven by the ability to form tumors from a small number of cells in immunodeficient mice, and the newly formed tumor usually shows histopathological characteristics similar to the original tumor. The frequency of cancer stem cells in a tumor can be estimated by limiting dilution analysis. Serial transplantation assay is also used to assess tumorigenic activity and self-renewal capacity of cancer stem cells.

4.3.2 Multilineage Potential

The well-known characters of normal tissue stem cells include multipotency as well as self-renewal capacity. Likewise, cancer stem cells undergo a process similar to differentiation under certain circumstances. In vitro incubation of breast cancer stem cells gives rise to cells positive for cytokeratin 8 (CK8) or mucin 1 (MUC1), both of which are breast epithelial cell markers. Glioma stem cells acquire similar cell morphology and marker expression pattern to astrocytes or oligodendrocytes when cultured in respective differentiation media. These results indicate that cancer stem cells have multilineage potential like normal tissue stem cells, although it is still controversial whether these processes can be defined as "differentiation." Thus, some researchers use the term "cancer-initiating cells" instead of "cancer stem cells" to literally and purely describe their ability to initiate tumor formation.

4.3.3 Resistance to Conventional Cancer Therapy

Another intriguing character of cancer stem cells is their resistance to conventional chemotherapy and radiation therapy (Dean et al. 2005; Baumann et al. 2008). In addition to their ability to self-renew, they are in the dormant phase and are quiescent and divide infrequently. Although our current therapeutic strategies against cancer succeed at eliminating rapidly proliferating bulk cells, they often miss slow-dividing cancer stem cells, which are the source of recurrence and metastasis.

Furthermore, cancer stem cells express high levels of specific ATP-binding cassette (ABC) drug transporters (Dean et al. 2005). The two ABC transporters that have been studied most extensively in cancer stem cells are ABCG2 (also known as breast cancer resistance protein 1, BCRP1) and ABCC2. By using the energy of ATP hydrolysis, these transporters actively efflux drugs from cancer stem cells, serving to protect them from cytotoxic agents (Gottesman et al. 2002).

Cancer stem cells contain lower levels of reactive oxygen species (ROS) than their matched bulk cancer cells (Diehn et al. 2009). Low ROS levels help to protect genomes in cancer stem cells from endogenous and exogenous ROS-mediated damage, including conventional radiation therapy. The mechanism for low ROS levels is at least partially due to the increased production of free radical scavengers. In addition, it has been reported that cancer stem cells show preferential checkpoint response and undergo DNA repair to counteract radiation damage (Bao et al. 2006a).

4.3.4 Invasion and Metastasis

The cancer stem cell model has shed new light on the mechanism of invasion and metastasis, and explain why, despite extensive intratumor heterogeneity, comparison of paired samples of primary tumors and distant metastases usually reveals

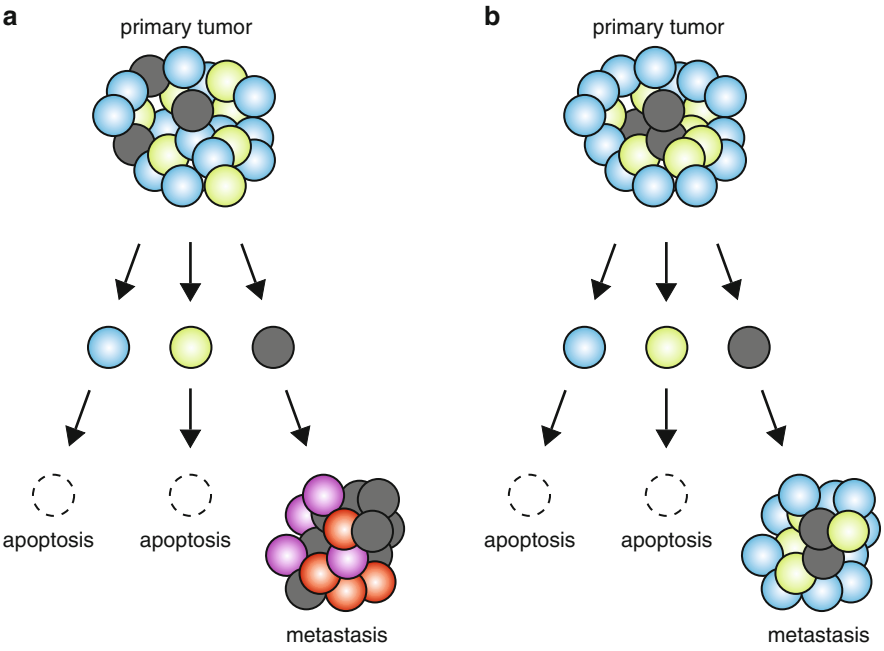

Fig. 4.2 (**a**) Based on the conventional stochastic cancer model, tumors are composed of heterogeneous mixtures of independent subclones with divergent genetic mutations. Different subclones are endowed with different functional properties and only selected clones (*dark gray*) can acquire ability to migrate and form metastases. The metastasis originates from a homogeneous monoclonal expansion of an individual subclone, which in turn can accumulate further mutations and diverge even further from the primary tumor. Therefore, the conventional stochastic cancer model predicts that primary tumors and corresponding metastases are substantially different. (**b**) In the cancer stem cell model, intratumor heterogeneity is mainly caused by cell differentiation and only cancer stem cells (*dark gray*) can migrate and form metastases. The cancer stem cells in metastatic sites undergo differentiation programs that closely resemble those observed in the corresponding primary tumors

striking similarities over a wide range of parameters, including tissue morphology (Brabletz et al. 2001), repertoire of genetic mutations (Losi et al. 1992; Khan et al. 2000; Zauber et al. 2003), and overall transcriptional profile as defined by gene expression arrays (Weigelt et al. 2003; Weigelt et al. 2005; D'Arrigo et al. 2005). These observations are not explained by the clonal evolution model, in which metastases are considered to originate from monoclonal expansions of specific individual tumor subclones endowed with specific genotypic and phenotypic features and therefore are postulated to be substantially different from primary tumors (Fig. 4.2a). However, under the cancer stem cell model, we can predict that, if two lesions share identical genetic abnormalities, they will also undergo similar differentiation programs and display similar patterns of intratumor heterogeneity (Fig. 4.2b).

In addition, C-X-C chemokine receptor type 4 (CXCR4) was reported to be highly expressed in prostate cancer stem cells (Dubrovska et al. 2012), suggesting the role of cancer stem cells as "pacemakers" of tumor metastasis.

4.4 Cancer Stem Cell Markers

Cancer stem cells have been prospectively isolated on the basis of their expression of particular markers such as CD44 and CD133 (Alison et al. 2010), although "non-cancer stem cells" defined as subpopulations negative for certain reported "cancer stem cell markers" also have tumorigenic activities in some contexts (Shmelkov et al. 2008; Quintana et al. 2010). Interestingly, cancer stem cells often share their markers with their normal counterparts, suggesting characteristic similarities between cancer stem cells and normal tissue stem cells.

Stem cells are known to efficiently extrude dyes such as Hoechst 33342. Cells with the capacity to efflux the dye were referred to as side population (SP) cells (Challen and Little 2006). SP cells have been identified in a variety of tissues, and they express high levels of stem-like genes and possess multi-potent differentiation potential (Goodell et al. 1996; Goodell et al. 1997; Pearce et al. 2004). The mechanism regulating the efflux of Hoechst dye can be explained, at least in part, by the expression of ABC transporters, including ABCG2 (Zhou et al. 2001).

SP cells have been also identified in a large variety of cancer cells (Kondo et al. 2004; Chiba et al. 2006; Haraguchi et al. 2006; Ho et al. 2007; Wang et al. 2007). When compared to non-SP population, SP cells isolated from cancer cells are highly enriched for the capacity to initiate tumor formation in immunodeficient mice. They also have the capacity to initiate tumors upon serial transplantation. Furthermore, SP cells in cancer cell population have increased expression of genes which are believed to be involved in the regulation of stem cell function, such as ABCG2 transporter. These data suggest that SP cells in tumors act as cancer stem cells.

4.5 Cancer Stem Cell Niche

Normal stem cells reside in a special microenvironment called "niche." They interact with the niche through adhesion molecules and exchange signals to maintain the specific features of stem cells (Wilson and Trumpp 2006; Martino and Pluchino 2006; Kiel and Morrison 2008; Wang and Wagers 2011). It has been suggested that there is a functional microenvironment to support cancer stem cells as well (Iwasaki and Suda 2009). This should also be considered a niche and is thus called "cancer stem cell niche."

Dick and colleagues demonstrated that anti-CD44 antibody-treated mice transplanted with AML cells exhibited a significantly lower rate of disease onset (Jin et al. 2006). In addition, Van Etten and colleagues showed that there was impaired

induction of chronic myelogenous leukemia (CML)-like myeloproliferative disease among recipient mice transplanted with BCR-ABL-transduced CML progenitors from CD44-mutant donors (Krause et al. 2006). These results indicate that CD44 is required for the homing and/or engraftment of leukemia stem cells to the niche, and that the binding of CD44-expressing leukemic stem cells to the niche is crucial for the maintenance of their tumorigenic activities. Interestingly, this role of CD44 in leukemia stem cells resembles that in normal hematopoietic stem cells, suggesting that cancer stem cells and normal stem cells share the maintenance system within their niches.

In brain tumor stem cell research, Gilbertson and colleagues revealed that brain tumor cells co-expressing Nestin and CD133 exist near the capillary vessels in the brain tumor (Calabrese et al. 2007). In addition, the CD133-positive subpopulation in human medulloblastoma developed brain tumors in a recipient nude mouse only when xenografted with endothelial cells. These results suggest that brain tumor stem cells rely on endothelial cells, which form a vascular niche to maintain the stem-like characters of brain tumor stem cells, such as self-renewal capacity and tumorigenic activity. Conversely, brain tumor stem cells themselves can elicit angiogenic effects by secreting factors such as vascular endothelial growth factor (VEGF) and stromal-derived factor 1 (SDF-1) (Bao et al. 2006b; Folkins et al. 2009). Furthermore, under certain conditions, brain tumor stem cells can even directly transdifferentiate into the endothelial lineage (Ricci-Vitiani et al. 2010; Wang et al. 2010), indicating a close connection between cancer stem cells and vascular niches.

4.6 Roles of TGF-β Signaling in Cancer Stem Cell Model

Cancer stem cells have been suggested to make use of a microenvironment similar to that found in normal stem cell niches for the maintenance of their stem cell-like properties. TGF-β signaling has been identified as a niche signal in the control of hematopoietic stem cells and hair follicle stem cells (Yamazaki et al. 2009; Oshimori and Fuchs 2012), and so a broader role for TGF-β signaling in the maintenance of cancer stem cells has been proposed. Recent studies have revealed crucial roles of TGF-β signaling in interaction between cancer stem cell and niche, as well as cancer stem cell-autonomous signaling pathways (Ikushima and Miyazono 2010a).

4.6.1 TGF-β Signaling in Breast Cancer Stem Cells

Breast cancer stem cells are, as described above, the first identified solid tumor stem cells (Dalerba et al. 2007b). Since the prospective identification using $CD44^{+}CD24^{-}$ subpopulation (Al-Hajj et al. 2003), the involvement of TGF-β signaling in breast cancer stem cells has been energetically studied. Recent reports have uncovered bilateral characters of TGF-β signaling in breast cancer stem cells: deprivation of

tumorigenic activities in some contexts and maintenance of stem cell-like characters in other contexts.

Wakefield and her colleagues showed that the suppression of the TGF-β pathway increased the size of the putative breast cancer stem cell compartment and promoted tumorigenesis by a mechanism that was independent of direct effects on proliferation (Tang et al. 2007). They demonstrated that the introduction of a dominant negative TGFBR2 enhanced the proliferation of immortalized and transformed human breast epithelial cell line, Ca1h, although the expression level of the cyclin-dependent kinase inhibitor p21 was unchanged. They also showed that TGF-β stimulation resulted in the loss of stem cell-like properties and the ability to form mammospheres. The ability of TGF-β to deprive breast cancer stem cells of tumorigenic activity was dependent on the down-regulation of Id1, which is highly expressed during embryogenesis and has been implicated in the regulation of self-renewal and differentiation. Id family proteins (Id1 through 4) act as inhibitors of differentiation and stimulators of cell growth by antagonizing the function of basic helix-loop-helix (bHLH) transcription factors (Miyazawa et al. 2002). Id1 has been reported to inhibit lineage commitment and differentiation in many cell types (Norton 2000) and to be a direct TGF-β target (Kang et al. 2003). These findings suggest that breast cancer stem cells benefit from similar mechanisms that regulate the function of normal stem cells.

Weinberg and his colleagues demonstrated that TGF-β signaling has an important role in the maintenance of stem cell-like properties and tumorigenic activity through the induction of epithelial–mesenchymal transition (EMT). A $CD44^{+}CD24^{-}$ subpopulation that was isolated from breast cancer specimens exhibited mesenchymal properties, and transformed mammary epithelial cells, in which EMT was induced by TGF-β stimulation, acquired stem cell-like properties, including mammosphere-forming ability (Mani et al. 2008; Scheel et al. 2011). Transformed mammary epithelial cells with TGF-β-induced EMT also showed higher tumorigenic activity in vivo and fewer cells were required to initiate tumor formation than cells without TGF-β treatment. These results connect TGF-β-induced EMT and the gain of epithelial stem cell properties of carcinoma.

4.6.2 TGF-β Signaling in Brain Tumor Stem Cells

Glioma stem cells are characterized by the expression of neural stem cell antigens (Singh et al. 2004; Kondo et al. 2004; Hirschmann-Jax et al. 2004) and share several characteristics with normal neural stem cells (Vescovi et al. 2006). The overexpression of TGF-β commonly seen in malignant glioma has been variously implicated in glioma cell proliferation, decreased apoptosis, migration, and tumor-specific immunosuppression (Golestaneh and Mishra 2005). TGF-β signaling also has important roles in the regulation of the stem cell properties of neural stem cells (Watabe and Miyazono 2009). These facts have shed some light on the role of TGF-β signaling in the maintenance of glioma stem cells.

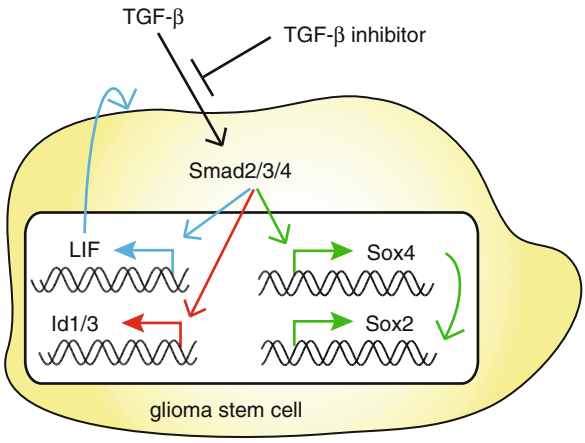

Fig. 4.3 TGF-β-Smad signaling pathway maintains stem cell-like properties and tumorigenic activity of glioma stem cells through many independent pathways, including the induction of the Sox4-Sox2 cascade, the activation of the LIF pathway, and the up-regulation of Id1 and Id3 expressions

TGF-β signaling has been reported to play pivotal roles in the maintenance of tumorigenic activity and stem cell-like properties of glioma stem cells (Peñuelas et al. 2009; Ikushima et al. 2009; Anido et al. 2010; Ikushima et al. 2011). TGF-β inhibitors markedly deprived glioma stem cells of glioma sphere-forming activity and self-renewal capacity in vitro and tumorigenic activity in vivo. Inhibition of TGF-β signaling also suppressed marker expressions that are associated with stem cell-like properties. These results indicate that microenvironmental niche-derived or glioma stem cell-autonomous TGF-β signaling maintains tumor-initiating abilities of glioma stem cells. TGF-β mediates this activity through the direct induction of the leukemia inhibitory factor (LIF) expression (Peñuelas et al. 2009). LIF activates the JAK-STAT pathway in glioma stem cells, leading to their increased tumorigenicity. Independently of this mechanism, TGF-β induces the expression of Sox2, a self-renewal gene that helps to maintain stem cell-like properties in embryonic stem cells and neural stem cells (Kamachi et al. 2000; Graham et al. 2003; Ferri et al. 2004). TGF-β induces the expression of Sox4, and this subsequently induces the expression of Sox2 (Ikushima et al. 2009; Ikushima et al. 2011). The maintenance of tumorigenic activity of glioma stem cells by TGF-β is also mediated by induction of Id1 and Id3 expressions (Anido et al. 2010). TGF-β signaling thus maintains the stem cell-like properties and tumorigenic activities of glioma stem cells through multiple pathways (Fig. 4.3), although interactions among these pathways have been still unclear.

4.6.3 TGF-β Signaling in Leukemia Stem Cells

TGF-β signaling is known to be a candidate niche signal in the control of hematopoietic stem cell hibernation (Yamazaki et al. 2009). Also, it plays important roles in the maintenance of leukemia stem cells.

CML is caused by a t(9;22)(q34;q11) translocation that generates a constitutively active tyrosine kinase, BCR-ABL (Ren 2005). BCR-ABL activates AKT signaling to suppress the forkhead O transcription factors (FOXO) in CML cells (Ghaffari et al. 2003; Essafi et al. 2005). In CML, a rare Lineage$^-$(Lin$^-$)Sca-1$^+$c-Kit$^+$ population was identified as leukemia stem cells (Hu et al. 2006; Neering et al. 2007; Zhao et al. 2009). Although the use of the tyrosine kinase inhibitor imatinib is a breakthrough for CML therapy, it does not deplete the leukemia stem cells, which drive the recurrence of CML (Graham et al. 2002; Michor et al. 2005; Roeder et al. 2006).

TGF-β signaling has crucial roles in the maintenance of leukemia stem cells in CML. TGF-β regulates AKT activation and FOXO3a localization in leukemia stem cells. Furthermore, this TGF-β-FOXO pathway maintains the stem cell-like properties and tumorigenic activities of leukemia stem cells (Naka et al. 2010). It was also demonstrated that a combination of TGF-β inhibition, FOXO3a deficiency, and imatinib treatment led to the efficient depletion of CML cells in vivo. These results indicate the central roles of TGF-β signaling in leukemia stem cells of CML. However, when leukemia stem cells were cultured with TGF-β inhibitors in a stroma-free system, colony formation was not inhibited (Naka et al. 2010), suggesting that the maintenance of leukemia stem cells depends not only on TGF-β produced by leukemia stem cells themselves but also on TGF-β in the surrounding microenvironment.

HOXA9 is involved in human AML caused by the translocation t(7;11)(p15;p15), through which HOXA9 gene is fused with the gene encoding NUP98, a nucleoporin protein (Nakamura et al. 1996; Borrow et al. 1996). Expression of the NUP98-HOXA9 fusion protein enforces expression of HOXA9 target genes and immortalizes hematopoietic myeloid progenitors, resulting in development of AML. In wild-type mouse hematopoietic stem cells transduced with NUP98-HOXA9, Smad4 binds to HOXA9 and inhibits nuclear transportation of HOXA9. In contrast, there is no cytoplasmic accumulation of HOXA9 in Smad4$^{-/-}$ hematopoietic stem cells, and as a consequence increased levels of HOXA9 is observed in the nucleus, leading to increased immortalization in vitro (Quéré et al. 2011). In addition, loss of Smad4 accelerates the development of NUP98-HOXA9-induced AML in vivo, and NUP98-HOXA9-transformed Smad4$^{-/-}$ leukemic cell population contains a higher amount of the leukemia stem cells than wild-type leukemia cells (Quéré et al. 2011). These results indicate that the cytoplasmic binding of Smad4 to HOXA9 is a mechanism to protect NUP98-HOXA9-induced transformation and acquisition of leukemogenic activity.

4.6.4 TGF-β Signaling in Gastric Cancer Stem Cells

Some molecules, including CD44 and aldehyde dehydrogenase 1 (ALDH1), have been reported as markers for gastric cancer stem cells (Takaishi et al. 2009; Katsuno et al. 2012). In addition, flow cytometric analyses using Hoechst 33342 have disclosed that SP cells in gastric carcinomas have higher tumorigenic activities, suggesting their roles as gastric cancer stem cells (Nishii et al. 2009; Ehata et al. 2011).

Mutations of TGFBR2, Smad4, and Smad2 have been reported to be responsible for progression of gastrointestinal tumors, indicating the tumor-suppressive activity of TGF-β signaling in gastrointestinal tumors (Wakefield and Roberts 2002; Bierie and Moses 2006). Besides suppression of cell proliferation and induction of apoptosis, TGF-β decreases the number of SP cells and so attenuates the tumor-forming ability of gastric cancer cells. TGF-β transcriptionally represses ABCG2 expression through direct binding of Smad2/3 to its promoter (Ehata et al. 2011). TGF-β also reduces the expression of ALDH1 and the size of the $ALDH1^+$ cell population in diffuse-type gastric cancer cells. In addition, ALDH1 expression inversely correlates with phosphorylation of Smad3 protein in human diffuse-type gastric cancer tissues (Katsuno et al. 2012). These results suggest that TGF-β reduces the cancer stem cell subpopulation through suppression of ABCG2 and ALDH1 expressions and inhibits tumor-initiating capacity of gastric cancer stem cells.

It has not yet been fully elucidated why TGF-β signaling suppresses tumorigenicity of gastric cancer stem cells while it maintains that of brain tumor stem cells and leukemia stem cells. Possible answers for this question may include the differential roles of TGF-β in the corresponding normal tissue stem cells (See Sect. 8).

4.6.5 TGF-β Signaling in Pancreatic Cancer Stem Cells

When incubated with TGF-β, pancreatic cancer stem cells enriched by sorting for SP cells change their shape into mesenchymal-like spindle-shaped appearance (Kabashima et al. 2009). This change is associated with significant reduction of E-cadherin expression and induction of Snail expression. Furthermore, SP cells show marked invasion activity in response to TGF-β treatment. Interestingly, such invasive activity is not induced by TGF-β stimulation in main population (MP) cells. These results suggest that TGF-β responsiveness is greater in SP cells than in MP cells, resulting in enhanced induction of EMT and invasiveness (Kabashima et al. 2009), although this study did not provide us direct evidences for the effect of TGF-β on tumorigenicity of pancreatic cancer stem cells.

4.7 TGF-β Signaling as Therapeutic Target in Cancer Stem Cell Model

According to the concept of cancer stem cells, therapeutic strategies that do not eradicate the cancer stem cell compartment are likely to achieve little success. They fail to prevent disease relapse and metastatic dissemination even when they might kill the majority of tumor cells and induce temporary regression of gross tumor lesions (Fig. 4.4). As mentioned above, cancer stem cells are considered to be inherently resistant to the toxic effect of conventional chemotherapeutic regimens and

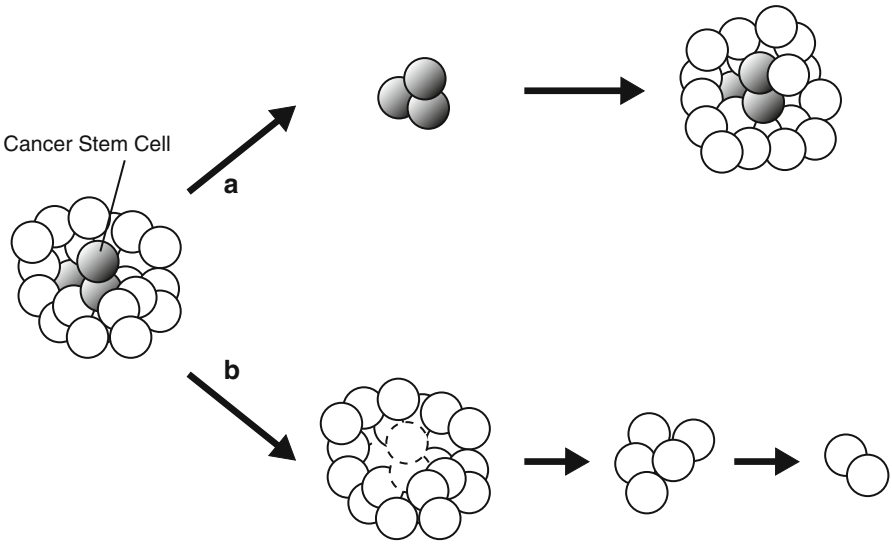

Fig. 4.4 Anti-tumor treatments designed for broad cytotoxic effects (**a**) may kill the majority of cancer cells within a tumor and induce dramatic regression of tumor masses. However, if a certain number of cancer stem cells are spared, tumor tissues can be regenerated, resulting in cancer recurrence. In contrast, anti-tumor treatments specifically designed to target cancer stem cells (**b**) might achieve long-term disease eradication by exhausting growth potential of cancer tissues, even though they are theoretically unable to cause rapid shrinkage of tumor lesions

radiation therapies. Therefore, investigational therapies should be developed, focusing on their ability to target the cancer stem cell subpopulation and its specific signaling pathways. However, there is one important problem in targeting signaling pathways specific for cancer stem cells: these pathways may also be important for normal stem cells. It has not yet fully determined what is the same or what is different between cancer stem cells and normal stem cells. Therefore, we should bear in mind that cancer stem cell-targeting agents could have adverse effects on maintenance of normal tissue stem cells.

The TGF-β pathway has been targeted using multiple strategies, including small-molecule inhibitors of the TGFBR1 kinase domain, TGF-β-specific neutralizing antibodies, and antisense compounds (Yingling et al. 2004). Some of them have been in clinical trials for human cancers (Schlingensiepen et al. 2006; Hau et al. 2007).

4.8 Concluding Remarks

As we have discussed, TGF-β is involved in the maintenance of the tumorigenic activity of cancer stem cells in several types of tumors in a tissue-specific manner. Targeting the pathways that maintain cancer stem cells might ultimately prove to be

an effective therapeutic strategy against malignant tumors. However, such pathways could have divergent roles in cancer stem cell populations from different patients.

Several in vitro and in vivo studies have uncovered cellular context-dependent diversity in TGF-β-induced cell responses (Ikushima and Miyazono 2010b). Because of such diversity, TGF-β can be both pro-tumorigenic and tumor-suppressive in a cellular context-dependent fashion. In addition, TGF-β promotes differentiation in some kinds of tissue stem cells and maintains stemness properties in others. Likewise, as we have discussed, TGF-β signaling shows positive effects on some kinds of cancer stem cells and negative effects on others. Such diversity among cancer stem cells could reflect both the differences between the oncogenic mutations expressed by the cells and their progeny and the differences in their origin. These differences will need to be taken into account when developing treatments based on TGF-β signaling for any individual patient. Also, we need to elucidate the exact mechanism by which TGF-β shows such complex effects against cancer stem cells in cellular context-dependent manner.

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Chapter 5
TGF-β as Tumor Suppressor: In Vitro Mechanistic Aspects of Growth Inhibition

Laurent Bartholin, David F. Vincent, and Ulrich Valcourt

Abstract Over time, TGF-β has emerged as both a tumor promoter and a tumor suppressor, depending on the cell type. Although it was first characterized as having the capacity to transform mesenchymal cells, many studies have shown it to exert an anti-proliferative effect on epithelial, endothelial, and immune cells. Most of the functional studies performed during the two decades following its discovery focused on its tumor-suppressive role and addressed a detailed understanding of the signaling events and molecular mechanisms by which TGF-β orchestrated cell growth arrest and apoptosis. This chapter entitled "TGF-β as tumor suppressor: in vitro mechanistic aspects of growth inhibition" depicts how TGF-β protects cells from transformation at the transcriptional, translational, and posttranslational levels. After a detailed view of the cytostatic program elicited by TGF-β to control the cell cycle, the focus shifts to TGF-β signaling pathways and biochemical events by which this cytokine governs the apoptotic program.

Keywords Apoptosis • Cell cycle regulation • Cytostasis • Posttranslational events • Smad signaling • Smad-independent pathways • Transcriptional regulation

Abbreviations

ATF/CREB	Activation transcription factor/cAMP responsive element-binding
bHLH	Basic helix-loop-helix
CDKs	Cyclin-dependent kinases

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ChIP	Chromatin immunoprecipitation
CKIs	Cyclin-dependent kinase inhibitors
CTGF	Connective tissue growth factor
eEF1A1	Eukaryotic elongation factor 1A1
eIF4E	Eukaryotic translation initiation factor-4E
EMT	Epithelial-to-mesenchymal transition
ERK	Extracellular signal-regulated kinase
FHBE	Forkhead-binding element
HDAC1	Histone deacetylase 1
HIPK2	Homeodomain-interacting protein kinase 2
IAP	Inhibitor of apoptosis
ID	Inhibitor of DNA binding/inhibitor of differentiation
INK4	Inhibitor of CDK4
JNK	c-Jun N-terminal kinase
MAP	Mitogen-activated protein
MAPK(K)	Mitogen-activated protein kinase (kinase)
MOMP	Mitochondrial outer membrane permeabilization
NGF	Nerve growth factor
PDGF	Platelet-derived growth factor
RB	Retinoblastoma protein
SBE	Smad binding element
TAK1	TGF-β-activated kinase 1
TGF-β	Transforming growth factor β
TIE	TGF-β inhibitory element
TIEG1	TGF-β-induced early gene 1
TNF	Tumor necrosis factor
TSS	Transcriptional start site
TβRI	TGF-β type I receptor
TβRII	TGF-β type II receptor
4E-BP1	eIF4E-binding protein 1

5.1 Introduction

Transforming growth factor-β (TGF-β) was first discovered as a tumor-promoting factor by supporting anchorage-independent growth of untransformed rat fibroblasts (Roberts et al. 1980; Roberts et al. 1981) and was further demonstrated to stimulate proliferation of different cell types in vitro such as mesenchymal-derived cells, like osteoblasts (Battegay et al. 1990), or glial cells, such as Schwann cells (Guenard et al. 1995; Ridley et al. 1989). However, the proliferative response induced by TGF-β in these cells appeared later on to be mostly mediated indirectly through autocrine secretion of other soluble factors, such as platelet-derived growth factor (PDGF) (Battegay et al. 1990; Leof et al. 1986; Soma and Grotendorst 1989) or connective tissue growth factor (CTGF) (Igarashi et al. 1993), and TGF-β has

then been proved to act mainly as a growth-inhibitory factor rather than a growth-promoting cytokine.

The TGF-β-mediated inhibition of cell growth was initially observed in vitro in a study focusing on the purification of growth inhibitors from the culture medium of BSC-1 epithelial kidney cells from African green monkey (Holley et al. 1980). Indeed, BSC-1-cell-conditioned medium was found to inhibit growth of Hs578T human mammary tumor cells (Holley et al. 1983), and TGF-β was identified as the mediator of this anti-proliferative effect (Tucker et al. 1984). It has been clearly established in the same period that TGF-β caused growth inhibitory responses in different cell types including epithelial (Masui et al. 1986; Tucker et al. 1984), endothelial (Baird and Durkin 1986; Frater-Schroder et al. 1986; Heimark et al. 1986), hematopoietic (Kehrl et al. 1986a; Kehrl et al. 1986b), neural cells as well as certain mesenchymal cells (reviewed in (Massague 1990)) and primary embryonic fibroblasts (Anzano et al. 1986). One of the first compelling evidence showing that TGF-β was able to inhibit cell growth in vivo was produced in 1987, when Silberstein et al. reported that implantation of ethylene vinyl acetate copolymer pellets allowing the slow release of TGF-β into small zones of the mammary gland could induce a potent inhibition of ductal growth (Silberstein and Daniel 1987).

In addition to its growth-inhibitory properties, the tumor-suppressive action of TGF-β was also shown to be mediated by its ability to induce apoptosis in different cell types both in vivo (Nguyen and Pollard 2000) and in vitro. Interestingly, TGF-β-mediated apoptosis concerns not only normal (Oberhammer et al. 1992) but also tumor cells (Lafon et al. 1996; Yanagihara and Tsumuraya 1992) and occurs in vitro concomitantly with the TGF-β-induced growth-inhibitory response in a cell culture (Kim et al. 1998; Rotello et al. 1991).

After the initial observation and characterization of the tumor-suppressive functions of TGF-β in the 1980s, the two last decades have been more dedicated to the detailed understanding of the signaling events and molecular mechanisms by which TGF-β orchestrated cell growth arrest and apoptosis. Hence, this chapter entitled "TGF-β as tumor suppressor: in vitro mechanistic aspects of growth inhibition" depicts how TGF-β protects cells from transformation at the transcriptional, translational, and posttranslational levels. After a detailed view of the cytostatic program elicited by TGF-β to control the cell cycle, the focus shifts to TGF-β signaling pathways and biochemical events governing the apoptotic program.

For a comprehensive review of mouse models with impaired TGF-β signaling and what they have taught us about the tumor-suppressive role of TGF-β, the reader is invited to read Chapter 6.

5.2 Control of the Cell Cycle: The Cytostatic Program

The growth-inhibitory response elicited by TGF-β has been extensively explored in epithelial cells. It involves many key cell-cycle regulators directly or indirectly regulated at the transcriptional level via the Smad signaling pathway or by

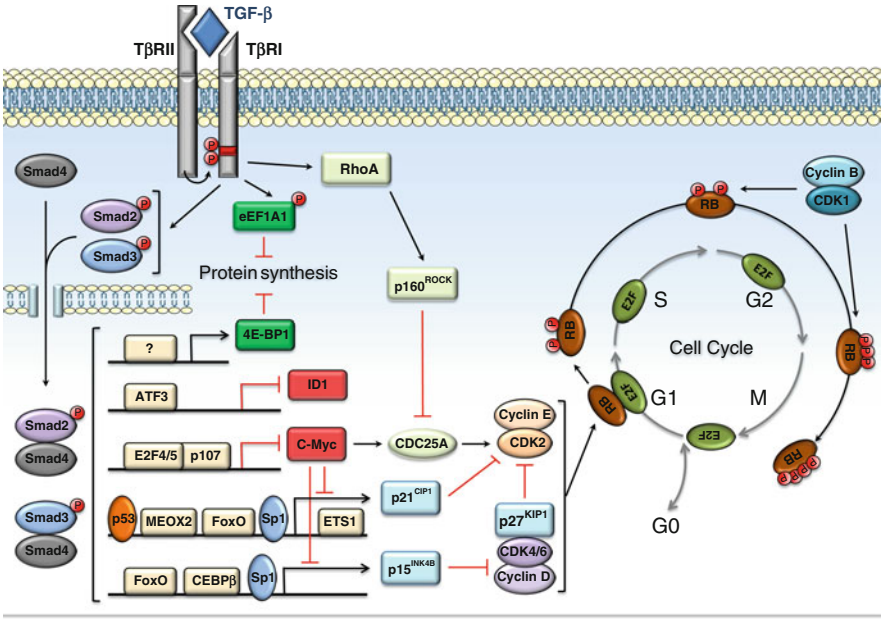

Fig. 5.1 The cytostatic program orchestrated by TGF-β. *Note*: The figure was illustrated using Servier Medical Art

posttranslational modifications mediated by the TGF-β receptor complex. The different targets of the TGF-β/Smad pathway thus cooperate to slow down the cell cycle and to eventually arrest cell growth, as is detailed in the following sections.

5.2.1 Control of the G1/S-Phase Transition by TGF-β

This growth inhibition is achieved by maintaining the retinoblastoma (RB) protein in a hypophosphorylated state (Laiho et al. 1990). Accordingly, ectopic expression of DNA virus oncoproteins sequestering the hypophosphorylated form of RB, such as the Adenovirus 5 protein ElA or the SV40 large T antigen, abolishes TGF-β-mediated growth inhibition (Pietenpol et al. 1990b). The requirement of a functional RB protein for TGF-β-mediated growth suppression has been confirmed in RB-deficient cells, in which TGF-β-mediated growth inhibition is abolished (Herrera et al. 1996). TGF-β inhibits cell growth by arresting the cell cycle at the early G1 phase (Holley et al. 1980; Laiho et al. 1990). Much effort has been devoted to understanding how TGF-β maintains the RB protein in a hypophosphorylated state and to identifying, cloning, and characterizing regulators of the cell cycle machinery (Fig. 5.1).

RB phosphorylation is catalyzed by cyclin-dependent kinases (CDKs), a family of serine/threonine protein kinases that are activated at specific points in the cell cycle. The enzymatic activity of CDKs requires the presence of cell-cycle-dependent regulators, the cyclins. Cell cycle progression from the G1 phase to the S phase is achieved through the successive action of the cyclin D-CDK4 and/or cyclin D-CDK6 and cyclin E/CDK2 complexes. Changes in cyclin levels, selective dephosphorylation of specific residues in CDKs, and interactions with stoichiometric inhibitors (the cyclin-dependent kinase inhibitors, CKIs) all contribute to tightly regulating the activity of these complexes (Sherr and Roberts 1999; Vermeulen et al. 2003). CKIs are classified into two families according to their mode of action: the CIP/KIP family and the INK4 (inhibitor of CDK4) family. The CIP/KIP family includes $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$, whereas the INK4 family comprises $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$, and $p19^{INK4D}$. The INK4 proteins prevent the interaction of cyclin D with CDK4/6. The CIP/KIP proteins can form ternary complexes with G1-phase cyclin-CDK complexes, but their role depends on the target: on the one hand, CIP/KIP proteins interact with cyclin E-CDK2 and inhibit its enzymatic activity by physically occluding the catalytic cleft of CDK2; on the other hand, they interact with and stabilize the cyclin D-CDK4/6 complex without necessarily inhibiting cyclin D-associated kinase activity (Sherr and Roberts 1999).

In epithelial cells, TGF-β has been shown to decrease the enzymatic activities of both cyclin D-CDK4/6 (Hannon and Beach 1994) and cyclin E-CDK2 (Koff et al. 1993). In Mv1Lu lung epithelial cells, it has also been shown to induce growth arrest by inhibiting CDK4 synthesis (Ewen et al. 1993). Extensive in vitro studies have been conducted, mostly on epithelial cells, in order to decipher the molecular mechanisms involved in the regulation of CDK activities and/or synthesis. Findings have led to identifying a limited number of factors involved in the growth-suppressive program governed by TGF-β, called the cytostatic program, which is shared by many lineages of epithelial origin in skin, lung, or mammary gland. The cytostatic program governed by TGF-β is orchestrated mainly at the transcriptional level, through the Smad transcription factors, which convey cues from the membrane to the nucleus. TGF-β-mediated transcriptional effects resulting in growth inhibition include (Fig. 5.1):

1. Transcriptional repression of the growth-promoting c-MYC transcription factor and of the differentiation-inhibiting ID nuclear factors (ID1 to −3).
2. Transcriptional activation of the genes encoding the cyclin-dependent kinase inhibitors $p15^{INK4B}$, $p21^{CIP1}$, and $p57^{KIP2}$.

TGF-β also exerts its cytostatic action via posttranslational events, such as protein modifications (phosphorylations) and changes in protein complex stoichiometry (stimulation of the inhibitory action of the $p27^{KIP1}$ CKI). The following paragraph presents the studies having led to major breakthroughs in this field.

5.2.2 *Smad-Mediated Transcriptional Repression of the* c-MYC *and* ID *Genes*

5.2.2.1 Regulation of the *c-MYC* Gene by the Smad Pathway

c-MYC was the first direct transcriptional target gene of TGF-β identified as being involved in the cell growth arrest induced by this cytokine in epithelial cells (Pietenpol et al. 1990a). In proliferating cells, *c-MYC* is transcriptionally active and its proto-oncoprotein product promotes the expression of genes essential to progression from the G1 to the S phase, such as those encoding cyclin D2 (Bouchard et al. 1999) or CDK4 (Hermeking et al. 2000). An important breakthrough in efforts to understand the cytostatic effect of TGF-β on epithelial cells was the finding that TGF-β can rapidly down-regulate *c-MYC* expression (Pietenpol et al. 1990a). Inversely, forced expression of *c-MYC* in epithelial cells impairs the cytostatic effect mediated by TGF-β (Alexandrow et al. 1995).

Repression of *c-MYC* transcription after TGF-β treatment is direct and involves the Smad signaling pathway. The activated Smad3/4 complex is recruited to a TGF-β inhibitory element (TIE) located between the two transcription start sites of the human *c-MYC* promoter, repressing its transcriptional activity (Chen et al. 2001). This event also involves the input of a repressive complex containing the E2F4 or E2F5 transcription factors and an additional co-repressor, the p107 pocket protein (Chen et al. 2002) (Fig. 5.1). Upon TGF-β stimulation and the subsequent phosphorylation of Smad3, the pre-formed Smad3-E2F4/5-p107 complex translocates into the nucleus, interacts with Smad4, and the Smad3/4-E2F4/5-p107 complex binds to the TIE of the *c-MYC* promoter (Chen et al. 2002; Frederick et al. 2004). The C/EBPβ transcription factor, which is a member of the basic leucine-zipper protein family, is also involved in TGF-β-mediated repression of *c-MYC* promoter activity, being recruited to the TIE sequence along with the Smad3/4-E2F4/5-p107 complex (Gomis et al. 2006b). TGF-β thus allows recruitment of a repressive transcriptional complex to the *c-MYC* promoter, causing cell cycle arrest through a rapid drop in the intracellular level of c-MYC protein.

5.2.2.2 Regulation of *ID* Genes by the TGF-β-Smad Pathway

Another set of target genes of the TGF-β/Smad signaling pathway has been implicated in the cytostatic response of epithelial cells: the ID (inhibitor of DNA binding) family of transcriptional regulators. ID proteins are inhibitors of cell differentiation belonging to the family of basic helix-loop-helix (bHLH) transcription factors (Perk et al. 2005). Classically, bHLH proteins bind to DNA as either homodimers or heterodimers and regulate gene transcription, either positively or negatively, depending on the dimer recruited to the DNA. As ID proteins lack a DNA-binding domain but have a dimerization domain, they act as dominant-negative proteins for other bHLH factors, preventing their binding to DNA and thus inhibiting their function (Perk et al. 2005).

The proteins ID1 to −3 are important positive regulators of cell growth (Fig. 5.1):

1. IDs indirectly promote inactivation of the RB protein by down-regulating CKI expression. They inhibit the transcriptional activity of bHLH proteins that positively regulate the CKI-encoding genes $p15^{INK4B}$, $p16^{INK4A}$, and $p21^{CIP1}$ by preventing their interaction with E-box DNA sequences contained in the promoters of CKI genes (Yokota and Mori 2002).
2. Alternatively, ID2 can block the cell-cycle-inhibiting action of retinoblastoma-family pocket proteins. This function correlates with the ability of ID2 to associate physically with active, hypophosphorylated forms of the pocket proteins (Lasorella et al. 1996; Lasorella et al. 2000). Like the E2F factors, ID2 can interact with the pocket domains of hypophosphorylated RB, p107, and p130. The formation of ID2-pocket protein complexes results in release of E2F from its repressive state, allowing progression of the cell cycle from G1 to S.

TGF-β causes a rapid decrease of ID1, −2, and −3 in various cell systems, such as mammary epithelial cells (NMuMG and MCF10A), normal epithelial lung cells (HPL-1), carcinoma-derived lung cells (A549), keratinocytes (HaCaT) (Kang et al. 2003; Kowanetz et al. 2004; Pardali et al. 2005; Siegel et al. 2003), and mouse embryonic fibroblasts (Lasorella et al. 2000). The mechanism by which TGF-β mediates this transcriptional repression is different for *ID1* and *ID2*.

The human *ID1* promoter contains three Smad binding elements (SBE) motifs downstream of an ATF/CREB binding site, suggesting transcriptional cooperation of Smad proteins with a member of the ATF/CREB (*activation transcription factor/cAMP responsive element-binding*) protein family (Kang et al. 2003). ATF-3, being rather a repressor than an activator of gene transcription, appears to be involved in TGF-β-mediated *ID1* gene repression. Interestingly, the Smad pathway directly regulates expression of the *ATF-3* gene, whose product interacts physically with the activated Smad3/4 complex and binds to the *ID1* promoter (Kang et al. 2003) (Fig. 5.1).

TGF-β-mediated regulation of *ID2* gene expression involves a different mechanism (Siegel et al. 2003). The *ID2* promoter contains E-box DNA sequences constituting a consensus binding site for bHLH transcription factors such as c-MYC. In proliferating cells, c-MYC has been shown to induce *ID2* expression through its recruitment to these E-box motifs (Lasorella et al. 2000), along with its partner Max (Siegel et al. 2003). Transcriptional repression of c-MYC and the subsequent drop in the c-MYC protein level might explain TGF-β-mediated inhibition of *ID2* gene transcription. Yet the time course of *ID2* down-regulation does not necessarily correlate with *c-MYC* repression in certain cell types (e.g., mammary epithelial cells), suggesting that other factors are required to negatively control *ID2* transcription. MAD proteins (MAD1-4) act as antagonists of c-MYC by replacing it in c-MYC-MAX transcriptional complexes. This leads to competition between complexes for the same regulatory sequences. TGF-β induces expression of MAD2/4 proteins in epithelial cells, causing increased association of MAD-MAX heterodimers with the *ID2* promoter (Siegel et al. 2003). Together, the TGF-β-mediated down-regulation of c-MYC and increased recruitment of MAD-MAX complexes to the *ID2* promoter contribute to the sustained transcriptional repression of this gene.

To our knowledge, the mechanism by which TGF-β causes transcriptional repression of *ID3* has not been elucidated to date. Unexpectedly, ID3 synthesis is reported to be induced upon TGF-β stimulation in B lymphocyte progenitor cells to mediate growth inhibition (Kee et al. 2001). This suggests that regulation of ID-factor expression by TGF-β might be cell- or context-specific.

5.2.3 *Transcriptional Activation of* $p21^{CIP1}$- *and* $p15^{INK4B}$- *Encoding Genes by Smad Complexes*

Repression of *c-MYC* and *ID* genes is generally not sufficient to ensure TGF-β-mediated growth inhibition and requires the activation of cell-cycle inhibitors. In epithelial cells, TGF-β induces a rapid increase in $p15^{INK4B}$ (Hannon and Beach 1994; Sandhu et al. 1997) and $p21^{CIP1}$ (Datto et al. 1995) protein levels, whereas in hematopoietic cells, it causes growth arrest through $p57^{KIP2}$ induction (Scandura et al. 2004). TGF-β directly stimulates transcriptional activation of the genes encoding the $p15^{INK4B}$, $p21^{CIP1}$, and $p57^{KIP2}$ CKIs through the Smad signaling pathway. The regulation of $p15^{INK4B}$ and $p21^{CIP1}$ promoter activity by the TGF-β/Smad pathway has been extensively analyzed, whereas the mechanism of TGF-β-mediated transcriptional regulation of $p57^{KIP2}$ is not yet fully understood.

Interestingly, the $p15^{INK4B}$ and $p21^{CIP1}$ promoters are similarly regulated. Under growth-promoting conditions, they are repressed by a complex containing c-MYC. This repressive complex must be released to allow recruitment of transactivators interacting with Smad proteins in TGF-β-stimulated cells. These transcriptional regulatory mechanisms are described below.

Forced expression of c-MYC blocks the transcriptional activation of $p15^{INK4B}$ (Warner et al. 1999) as well as the induction of $p21^{CIP1}$ (Claassen and Hann 2000) in epithelial cells, suggesting that c-MYC may hinder transcriptional activation of the genes encoding these CKIs. This appears to be the case, since in proliferating cells, c-MYC is expressed to a high level and tethered to the $p21^{CIP1}$ and $p15^{INK4B}$ promoters via the zinc finger protein Miz-1, thus interfering with transcriptional activation by TGF-β (Seoane et al. 2001; Seoane et al. 2002; Staller et al. 2001). In the $p15^{INK4B}$ promoter, the c-MYC-Miz-1 complex binds directly to the transcriptional initiator element to block transcriptional activation (Staller et al. 2001). It is noteworthy that c-MYC-mediated repression of $p21^{CIP1}$ occurs in other contexts besides TGF-β signaling. Binding of the c-MYC-Miz-1 complex also impedes transcriptional activation of $p21^{CIP1}$ by other transactivators, such as p53 (in the context of the response to DNA damage) (Seoane et al. 2002).

In this context, c-MYC down-regulation mediated by TGF-β signaling (Fig. 5.1) is essential to rendering the $p21^{CIP1}$ and $p15^{INK4B}$ promoters competent for transcriptional activation. Yet relief from inhibition by c-MYC is not sufficient for activation of these promoters. Transcriptional activation of both promoters by TGF-β requires the input of other transactivators. A precise analysis of the $p21^{CIP1}$ and $p15^{INK4B}$

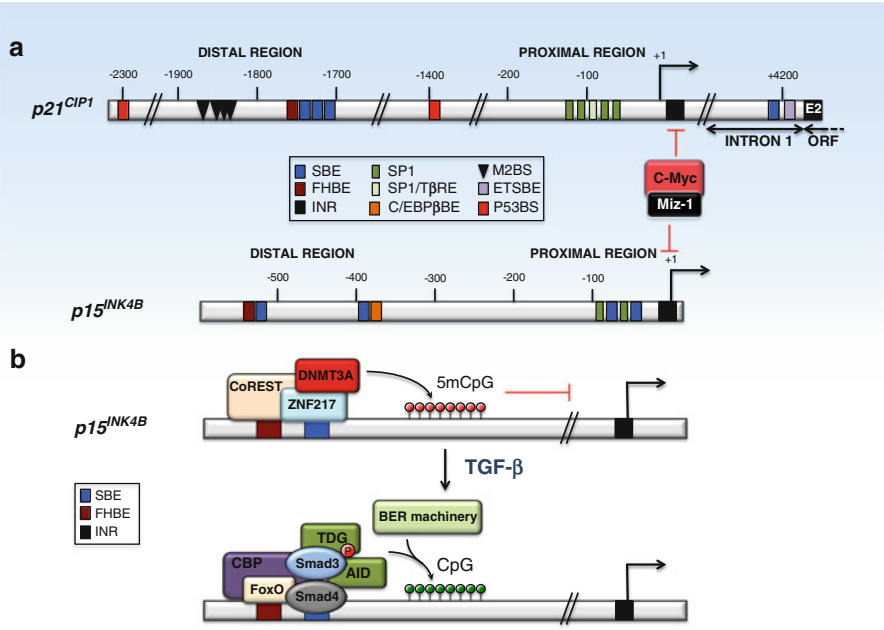

Fig. 5.2 (**a**) Regulatory elements of the $p15^{INK4B}$ and $p21^{CIP1}$ promoters involved in the TGF-β/Smad-mediated transcriptional control. (**b**) Active demethylation of the $p15^{INK4B}$ promoter in response to the TGF-β/Smad signaling pathway. SBE, Smad-binding element; FHBE, Forkhead-binding element; Sp1, Sp1-binding site; TβRE, TGF-β-responsive element; INR, transcriptional initiator element; ETS, ETS-binding element; P53BS, p53-binding site; M2BS, MEOX2-binding site (putative); C/EBPβBE, C/EBPβ-binding element. *Note*: The figure was illustrated using Servier Medical Art

promoters of different species has contributed to identifying the transcriptional regulators that orchestrate transcriptional activation in response to TGF-β.

The mechanisms involved would appear to be the same for $p21^{CIP1}$ and $p15^{INK4B}$ and to be shared by many epithelial cells:

1. The $p21^{CIP1}$ (Moustakas and Kardassis 1998) and $p15^{INK4B}$ (Feng et al. 2000) genes contain not only a GC-rich rich region in the proximal promoter region but also SBEs in more distal enhancer elements (Gomis et al. 2006a; Seoane et al. 2004), where activated Smad3/Smad4 complexes can bind upon TGF-β stimulation (Fig. 5.2a).
2. The SBEs in the distal enhancer elements are located close to a consensus site for binding of forkhead-family proteins. The $p21^{CIP1}$ promoter contains one forkhead-binding element (FHBE) adjacent to three closely spaced SBEs, whereas the $p15^{INK4B}$ promoter contains an FHBE followed by one SBE (Fig. 5.2a). Upon TGF-β stimulation, activated Smad3/4 forms a complex with FoxO proteins (FoxO1, FoxO3, and FoxO4), recruiting them to the promoters and thus activating transcription (Gomis et al. 2006a; Seoane et al. 2004).

3. The transcriptional activator complex also involves Sp1 transcription factors, interacting with Smad proteins in the proximal regions of both promoters (Fig. 5.2a). In the *p15*^{INK4B} promoter, two Sp1 binding sites are followed by one SBE (Feng et al. 2000), whereas the GC-rich proximal part of the *p21*^{CIP1} promoter contains one SBE and is flanked by several Sp1 binding sites (Moustakas and Kardassis 1998; Pardali et al. 2000). In this particular region, interestingly, a Smad2/3-Sp1 complex also recruits the c-MYC factor (Feng et al. 2002) to the *p15*^{INK4B} promoter, suggesting that in the case of this promoter at least, the Sp1 complex might be involved in both positive and negative regulatory mechanisms (in the latter case, via the c-MYC-Miz-1 complex) (Feng et al. 2002). Accordingly, the transcriptional repression mediated by c-MYC is lost when the Sp1-binding sites are mutated. Conversely, when c-MYC-mediated repression is relieved upon TGF-β signaling, Miz-1 may cooperate with the Smad-Sp1 complex to elicit full activation of *p15*^{INK4B} (Seoane et al. 2001). Taken together, these findings suggest that Sp1-binding elements serve as docking sites for both repressor and activator complexes.
4. The *p15*^{INK4B} promoter (but not the *p21*^{CIP1} promoter) contains a third TGF-β-responsive region, in its distal part, where an SBE is immediately followed by a C/EBPβ binding site (Fig. 5.2a). Upon stimulation, a Smad3/4-C/EBPβ complex is recruited to this promoter region to enable transcriptional activation. As a regulator of both c-MYC repression and p15^{INK4} activation, C/EBPβ appears as a crucial mediator of the cytostatic program governed by TGF-β (Gomis et al. 2006b).
5. p53, p63, and p73—major transactivators of *p21*^{CIP1} expression—have also been implicated in TGF-β-mediated transcriptional activation of the *p21*^{CIP1} promoter. Indeed, down-regulation of p53 or p63 in epithelial cells abolishes *p21*^{CIP} gene induction by TGF-β (or activin). The p53 transactivator interacts with activated Smads at the distal region of the *p21*^{CIP} promoter to promote robust induction of *p21*^{CIP} expression (Cordenonsi et al. 2003) (Figs. 5.1 and 5.2).
6. The induction of *p21*^{CIP1} expression in response to TGF-β also requires the involvement of MEOX2 (mesenchyme homeobox2). *MEOX2* is a target gene of the TGF-β signaling pathway, whose product has been shown to interact physically with Smad3 and Smad4 and to bind to the distal region of the *p21*^{CIP1} promoter (Figs. 5.1 and 5.2) (Valcourt et al. 2007).
7. Chromatin immunoprecipitation (ChIP) experiment followed by microarray (ChIP on chip) analysis identified two strong Smad-binding regions in the first intron of the *p21*^{CIP1} gene downstream from the translation start site (Figs. 5.1 and 5.2). The binding of Smad2/3 proteins in those intronic regions is strengthened by the presence of the ETS1 factor, the v-ets erythroblastosis virus E26 oncogene homolog (Koinuma et al. 2009).
8. Both *p15*^{INK4B} and *p21*^{CIP1} promoters contain a CpG island within 1 kb of the transcriptional start site (TSS). In proliferating cells, core CpG islands are highly methylated contributing to the silencing of the two promoters. Upon stimulation, TGF-β rapidly triggers an active demethylation of both promoters (Thillainadesan et al. 2012). In the case of the *p15*^{INK4B} promoter, the CpG island is located

adjacently to SBE/FHBE sequences (Fig. 5.2b). The promoter methylation and subsequent gene silencing is mediated by the constitutive binding of a repressive complex consisting of the oncogene ZNF217, coREST (corepressor of REST), and the DNA methyltransferase DNMT3A. TGF-β induces the displacement of the CoREST-ZNF217-DNMT3A complex, and the subsequent recruitment of the activated Smad3/4 proteins, together with the CBP co-activator and enzymes regulating DNA methylation. Indeed, the Smad3/4 complex recruits the cytidine deaminase AID and the DNA glycolases TDG and MBD4, as well as components of the base excision repair (BER) machinery (Thillainadesan et al. 2012) (Fig. 5.2b). It is important to highlight that this observation is the first showing that epigenetics is a dynamic process that could be regulated by intracellular signaling molecules.

The cytostatic activity of TGF-β also results from additional cooperation between Smad and other signaling pathways. For instance, TGF-β induces Notch signaling to mediate $p21^{CIP1}$ expression (Niimi et al. 2007). Indeed, the TGF-β/Smad pathway induces the expression of the Notch transmembrane ligand Jagged1, which in turn triggers the Notch intracellular signaling pathway in adjacent cells. This process ultimately stimulates the expression of the $p21^{CIP1}$ gene, through the activity of the CLS transcription factor, an effector and a partner of the Notch intracellular signaling domain.

In hematopoietic cells, TGF-β induces robust expression of $p57^{KIP2}$ rather than $p21^{CIP1}$ and $p15^{INK4B}$ (Scandura et al. 2004). TGF-β-mediated up-regulation of $p57^{KIP2}$ expression is direct and requires the action of the Smad3/4 complex on a proximal region of the $p57^{KIP2}$ promoter. Surprisingly, this promoter contains no consensus SBE but includes putative binding sites for other transcription factors, such as YY1, RUNX1, and Sp1, known to interact physically with Smad proteins. Whether these factors cooperate with Smads in regulating $p57^{KIP2}$ expression in hematopoietic cells remains to be elucidated.

Thus, TGF-β/Smad signaling involves (1) several transcription factors, acting both at the proximal promoter and at distal enhancer elements, as well as (2) the DNA demethylation machinery, to mediate rapid and sustained expression of both $p21^{CIP1}$ and $p15^{INK4B}$. The functional consequence of $p15^{INK4B}$, $p21^{CIP1}$, and $p57^{KIP2}$ induction is the abolition of CKD4/6 and CDK2 kinase activities, causing an arrest of G1-to-S cell cycle progression by impairing RB phosphorylation.

5.2.4 Stimulation by TGF-β of the $p27^{KIP1}$ Inhibitory Function

In growth-stimulated epithelial cells, $p27^{KIP1}$ interacts with active cyclin D-CDK4/6 complexes, and its expression level is not altered by TGF-β in the course of the cell cycle (Reynisdottir et al. 1995). TGF-β regulates $p27^{KIP1}$ activity by increasing its bioavailability for binding to the cyclin E-CDK2 complex. By increasing the pool of $p15^{INK4B}$ protein, TGF-β promotes the formation of $p15^{INK4B}$-CDK4/6 complexes

at the expense of ternary $p27^{KIP1}$-cyclin D-CDK4/6 complexes, and thus causes $p27^{KIP1}$ release. This leads to the recruitment of $p27^{KIP1}$ to available cyclin E-CDK2 complexes, which become inhibited (Reynisdottir et al. 1995). Together with the concomitant induction of $p21^{CIP1}$, the displacement of $p27^{KIP1}$ protein from active cyclin D-CDK4/6 to cyclin E-CDK2 complexes ensures robust inhibition of CDK2 catalytic activity, prevents phosphorylation of RB, and causes cell cycle arrest at the G1-to-S transition (Fig. 5.1).

5.2.5 *TGF-β-Mediated Down-Regulation of CDC25A Prevents CDK2 Activation*

TGF-β also regulates the activity of the G1-phase CDKs through posttranslational modifications. The activity of CDKs is negatively regulated by their phosphorylation on specific threonine (Thr14) or tyrosine (Tyr15) residues (Donzelli and Draetta 2003). In the G1 phase, the CDK dual-specificity phosphatase CDC25A dephosphorylates CDK2, allowing subsequent activation of cyclin E-CDK2 complexes (Donzelli and Draetta 2003). This activation step is crucial to subsequent cell cycle progression to the S phase. TGF-β has been shown to decrease the expression of CDC25A in epithelial cells, both in vitro, in cultured mammary epithelial cells (Iavarone and Massague 1997), and in vivo, in mice characterized by specific overexpression of TGF-β1 in hepatocytes (Bouzahzah et al. 2000). More precisely, TGF-β causes direct transcriptional repression of the *CDC25A* gene through recruitment of an E2F4-p130 repressive complex, together with the histone deacetylase 1 (HDAC1), to a region located immediately upstream of the transcription start site of the *CDC25A* promoter (Bouzahzah et al. 2000; Iavarone and Massague 1999). Furthermore, the proto-oncogene c-MYC has been shown to cooperate with its partner Max to directly activate transcription of *CDC25A* (Galaktionov et al. 1996). In this context, TGF-β might also regulate indirectly the transcriptional repression of *CDC25A*, by facilitating Smad-mediated transcriptional repression of the c-*MYC* gene. Alternatively, another TGF-β-dependent mechanism might negatively regulate CDC25A activity. TGF-β activates the RhoA-$p160^{ROCK}$ axis, causing an inhibitory phosphorylation of CDC25A (Bhowmick et al. 2003). In conclusion, CDC25A is targeted by TGF-β via both transcriptional and posttranslational events so as to inhibit CDK2 activation (Fig. 5.1).

5.2.6 *TGF-β-Mediated Inhibition of the Translational Machinery is Involved in Cell-Growth Arrest*

Protein synthesis is a tightly regulated process involving more than a hundred of different proteins. Regulators of the translational machinery have emerged as new players in cell-autonomous tumor-suppressive function of TGF-β. Two studies have

linked actors of the translation process to the cytostatic program governed by TGF-β. As detailed below, the first mechanism involves transcriptional activation of the translation-inhibiting protein 4E-BP1 (eIF4E-binding protein 1), mediated by the Smad signaling pathway (Azar et al. 2009); the second relies on catalytic inactivation of the translation initiation factor eEF1A1 (eukaryotic elongation factor 1A1) by the TGF-β type I receptor (TβRI) (Lin et al. 2010) (Fig. 5.1).

The translation-inhibiting protein 4E-BP1 blocks the function of the cap-binding eukaryotic translation initiation factor-4E (eIF4E), which docks the ribosome at the 5′-end of mRNA in order to initiate protein synthesis. 4E-BP1 is required for TGF-β-mediated growth arrest in pancreatic carcinoma cells, mouse embryonic fibroblasts, and keratinocytes (Azar et al. 2009). The gene encoding 4E-BP1 appears as a direct target of TGF-β signaling, its transcription being activated by TGF-β. Smad4 has been shown to bind to a conserved SBE located in the first exon of this gene. Artificial down-regulation of *4E-BP1* expression abolishes TGF-β-induced cell cycle arrest in pancreatic epithelial cells and embryonic fibroblasts (Azar et al. 2009). Thus, by increasing the level of 4E-BP1, TGF-β contributes to inhibiting the translational machinery and to causing cell growth arrest.

From a phage library screen followed by phosphorylation assays performed with a soluble form of recombinant TGF-β type I receptor, the eukaryotic elongation factor 1A1 (eEF1A1) has emerged as a novel direct substrate of TβRI kinase activity (Lin et al. 2010). TβRI phosphorylates eEF1A1 at serine 300, an amino acid crucial to efficient binding of amino-acyl-tRNA to the elongation factor. In vitro, TGF-β-mediated phosphorylation of serine 300 causes efficient inhibition of protein synthesis and subsequent growth arrest in the breast cancer cell line MCF-7 (Lin et al. 2010). This is the first evidence of a direct link between TGF-β receptors and the regulation of protein synthesis in the control of cell proliferation.

TGF-β has recently been shown to regulate epithelial-to-mesenchymal transition (EMT) by stimulating the translation elongation of selected transcripts (Hussey et al. 2011). More precisely, the translation of several genes essential for EMT is inhibited by the heterogeneous nuclear ribonucleoprotein E1 (hnRNP E1), which blocks eEF1A1 release during translation elongation. Phosphorylation of hnRNP E1 in response to TGF-β signaling disrupts the hnRNP E1-eEF1A1 interaction, triggering EMT. Whether a similar mechanism is employed by TGF-β in order to regulate cytostasis has not been investigated yet.

5.3 The Apoptotic Program

5.3.1 Overview of Apoptotic Pathways

Apoptosis or programmed cell death (Conradt 2009; Joza et al. 2002) is a process leading to ordered disruption of the cell without leakage of cell components. It is absolutely required both during embryogenesis (elimination of interdigital structures, regulation of neuron cell numbers) and in adult life (destruction of immature

lymphocytes, regulation of regenerative growth of the liver, involution of the mammary gland after lactation). Apoptosis is characterized by activation of several pathways leading to activation of caspase-family cysteine proteases (Brenner and Mak 2009; Kroemer et al. 2007). Caspases are synthesized as pro-enzymes requiring activation in order to trigger apoptosis.

Two caspase apoptotic pathways have been described:

1. Extrinsic apoptosis is a form of cell death induced by extracellular signals resulting in binding of ligands to specific transmembrane receptors of the tumor necrosis factor/nerve growth factor (TNF/NGF) family, called death receptors. Upon ligand binding, receptor oligomerization allows recruitment of adaptor proteins and subsequent formation of a death initiation signaling complex (DISC) causing activation of the caspase cascade (initiated by caspase-8) (Lavrik and Krammer 2012).
2. The intrinsic pathway is activated in response to internal stresses, such as DNA damage and accumulation of free oxidant species. The various stress-induced signals converge on the mitochondria and determine mitochondrial outer membrane permeabilization (MOMP), which in turn results in dissipation of the mitochondrial membrane potential and hence the release of proteins, such as cytochrome C and the apoptosis-inducing factor (AIF), contributing to caspase-9 activation. The local regulation and occurrence of MOMP involve proteins of the BCL-2 family, which comprises pro-apoptotic factors (BAX, BAD, BIM) whose activity is finely tuned by its anti-apoptotic members (BCL-2, BCL-XL) (Green and Kroemer 2004).

5.3.2 TGF-β-Mediated Apoptosis

TGF-β-induced apoptosis plays important roles in many in vivo biological processes, such as wound healing (Amendt et al. 2002) and involution of the mammary epithelium after lactation (Nguyen and Pollard 2000) (see Chapter 6). It also plays a crucial role in tumor suppression, by eliminating cells with a malignant potential (Guasch et al. 2007; Yang et al. 2006). In vitro, a pro-apoptotic activity of TGF-β has been observed in many different cell types, such as B and T lymphocytes, prostate, mammary, and lung epithelial cells, hepatocytes, and neurons (reviewed in (Schuster and Krieglstein 2002)). Yet TGF-β has also been shown to promote survival of highly differentiated cells, such as cells of the nervous system (Sanchez-Capelo 2005).

TGF-β-induced apoptosis occurs mainly via the mitochondrial pathway (Schuster and Krieglstein 2002) (Fig. 5.3). In cell types where TGF-β (or activin) induces an apoptotic event, programmed cell death is usually associated with (1) down-regulation of anti-apoptotic factors such as BCL-2 or BCL-XL and/or (2) up-regulation of apoptosis-inducing factor such as BAX or BIK (Saltzman et al. 1998; Schuster and Krieglstein 2002). In contrast to the TGF-β cytostatic program, which

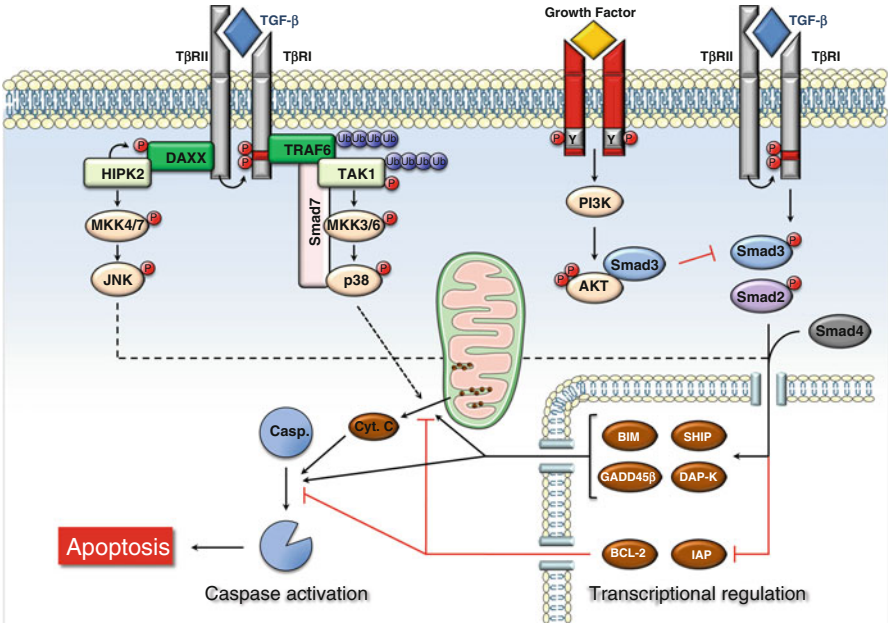

Fig. 5.3 The apoptotic program in response to TGF-β. *Dashed arrows* represent indirect biochemical events. *Note*: The figure was illustrated using Servier Medical Art

is shared by many cell types and involves a limited set of genes and regulators, the TGF-β apoptotic program is mostly cell-type specific and involves a plethora of regulators. It is also less well known and understood.

TGF-β regulates apoptosis via two distinct means that are not mutually exclusive (Fig. 5.3):

1. Smad-dependent transcription control of pro-apoptotic genes whose products are directly involved in the death pathway.
2. Smad-independent pathways initiated by enzymatic or non-catalytic activities of the TGF-β receptor complex.

The following sections present selected studies illustrating the variety of mechanisms that TGF-β can regulate to trigger cell death.

5.3.2.1 The Apoptotic Gene Program Regulated by the Smad Pathway

One of the first evidence of Smad pathway involvement in TGF-β-triggered apoptosis was the identification, in 1997, of the TGF-β-induced early gene 1 (TIEG1) as an immediate target gene of the transcription-regulating action of TGF-β. TIEG1 is an Sp1/Krüppel-like zinc finger transcription factor shown to induce apoptosis in pancreatic epithelial cells (Tachibana et al. 1997), mink lung epithelial cells (Chalaux

et al. 1999), and hepatocytes (Ribeiro et al. 1999). The mechanism by which TIEG induces apoptosis is believed to rely on generation of reactive oxygen species, which would be the initiating death signals leading to a decreased BCL-2 protein level and subsequent loss of mitochondrial membrane potential.

In hematopoietic cells, TGF-β induces expression of the inositol phosphatase SHIP (Src homology 2 (SH2) domain-containing 5′-inositol phosphatase), a central regulator of phospholipid metabolism. Smad-mediated transcriptional activation of the *SHIP* gene results in inhibition of an activating phosphorylation of the survival factor AKT (also known as protein kinase B, PKB), associated with subsequent cell death. Artificial down-regulation of SHIP compromises TGF-β-triggered death of myeloma cells (Valderrama-Carvajal et al. 2002).

Upon TGF-β stimulation, the Smad2/3-4 complex activates transcription of the gene encoding the death-associated protein kinase (DAP-K), an enzyme directly involved in regulating apoptosis. By facilitating cytochrome C release from the mitochondria and dissipation of the mitochondrial membrane potential, TGF-β-induced DAP-K expression causes apoptosis of Hep3B hepatocellular carcinoma cells (Jang et al. 2002).

TGF-β stimulates transcription of GADD45β, a member of the stress- and cytokine-induced factor family, via the action of activated Smad3/4 complex on the proximal region of the *GADD45β* promoter (Yoo et al. 2003) and on an enhancer element located in the third intron (Major and Jones 2004). GADD45β was first identified in a complex containing the p38-activating kinase MEKK4/MTK1 (a mitogen-activated protein (MAP) kinase kinase kinase). By phosphorylating the MAPKK MKK3/6, the GADD45β-MEKK4 complex promotes activation of the MAPK p38, thus regulating p38-mediated biological functions such as apoptosis (Takekawa et al. 2002). In hepatocytes, the involvement of the p38 signaling pathway in TGF-β/GADD45β-triggered apoptosis is supported by the fact that apoptosis is inhibited by a small-molecular-weight inhibitor selectively blocking p38 kinase activity (Yoo et al. 2003). Expression of GADD45β in hepatocytes is sufficient to trigger apoptosis in the absence of TGF-β stimulation, whereas down-regulation of GADD45β expression inhibits TGF-β-induced p38 activation and apoptosis (Yoo et al. 2003).

The Smad pathway has been shown to control directly or indirectly the transcriptional expression of BCL-2-family members, key regulators of the intrinsic apoptotic pathway. A first example concerns down-regulation of the prototypic anti-apoptotic protein BCL-2 by TGF-β in vivo. Ectopic expression of Smad3 in transgenic mice protects the liver from chemically induced carcinogenesis (Yang et al. 2006). Indeed, Smad3 exerts its protective role by inducing apoptosis in hepatocytes in order to prevent their malignant conversion. Smad3 triggers apoptosis by down-regulating the transcription of the gene encoding the anti-apoptotic protein BCL-2. This repression is exerted upon binding of a Smad3-containing transcriptional complex to a GC-rich TGF-β-responsive element in the *Bcl-2* promoter (Yang et al. 2006). Interestingly, Smad3-mediated apoptosis occurs concomitantly with p38 phosphorylation in both liver tumors of transgenic mice and primary hepatocytes cultured in the presence of TGF-β (Yang et al. 2006). As p38 MAPK activity

appears also to be involved in TGF-β-mediated apoptosis (see above), these findings indicate that Smad-dependent and Smad-independent pathways may cooperate in the same cell to strengthen death-initiating cues.

While down-regulating anti-apoptotic factors, TGF-β also positively regulates pro-apoptotic factors of the BCL-2 family. For example, it increases the level of the pro-apoptotic BIM protein in B lymphocytes (Ramesh et al. 2008; Wildey et al. 2003) and hepatocytes (Wildey and Howe 2009) in a Smad3-dependent manner so as to trigger apoptosis via the mitochondrial pathway. TGF-β can control the BIM protein level via two distinct mechanisms:

1. The canonical TGF-β/Smad3 pathway indirectly regulates transcriptional activation of the *BIM* gene, inducing expression of transcription factor RUNX1, which cooperates with FoxO3 and binds to the *BIM* promoter to trigger transcriptional activation in hepatocytes (Wildey et al. 2003). Despite the presence of a putative SBE in the *BIM* promoter, cooperation of activated Smad3/4 with the RUNX1-FoxO3 complex has not yet been established.
2. TGF-β indirectly promotes BIM protein stabilization. Extracellular signal-regulated kinase (ERK)-regulated phosphorylation of BIM targets it for degradation by the proteasome machinery. This posttranslational event is inhibited by the MAPK phosphatase MKP2, which dephosphorylates and thus inactivates the ERK kinase. The *MKP2* gene responds directly to TGF-β. By stimulating transcriptional activation of *MKP2*, the Smad3/4 complex causes stabilization of the BIM protein (Ramesh et al. 2008).

Survivin, a member of the mammalian inhibitor of apoptosis (IAP) family of endogenous caspase inhibitors, is also transcriptionally down-regulated by TGF-β (Yang et al. 2008). Interestingly, TGF-β represses transcription o*f* the *Survivin* gene by a peculiar mechanism. The proximal region of the *Survivin* promoter contains two canonical SBEs and two cell cycle repressor elements: a cell-cycle-dependent element (CDE) and a cell-cycle gene homology region (CHR). Both the CDE and the CHR are indispensable to TGF-β-mediated transcriptional repression in reporter assays. TGF-β causes recruitment of an RB-E2F4 repressive complex to the CDE/CHR elements of the *Survivin* promoter. Although Smad2/3 signaling is absolutely required for TGF-β-mediated repression of *Survivin* and although Smad3 binds to the minimal proximal region of the *Survivin* promoter, these SBEs do not seem to be the key elements mediating repression, since Smad4 does not bind to this particular promoter region and since mutations within the two SBEs do not abolish TGF-β-mediated transcriptional repression in reporter assays. Smad proteins might be required in this transcriptional repression indirectly, for their ability to maintain the RB protein in a hypophosphorylated state in a complex with E2F4 factors (in the framework of the TGF-β-governed cytostatic program). Alternatively, a Smad complex might interact physically with unidentified SBEs located elsewhere in the promoter or in other regulatory elements (Yang et al. 2008).

AKT overexpression is sufficient to block TGF-β-mediated apoptosis, independently of AKT kinase activity (Conery et al. 2004; Remy et al. 2004). This inhibition results from direct interaction of AKT with Smad3, preventing phosphorylation of

Smad3 by TβRI (titration). Hence, the Smad3:AKT ratio is a crucial determinant of the sensitivity to apoptosis in response to TGF-β, stronger apoptotic responses being observed in cells with higher Smad3:AKT ratios (Conery et al. 2004). More interestingly, the growth of all cell lines tested is potently inhibited by TGF-β, while their response to apoptosis depends on the Smad3-to-AKT ratio (Conery et al. 2004). The AKT-Smad3 complex might be part of a regulatory mechanism enabling a given cell to switch from the cytostatic program to the apoptotic program in response to TGF-β (Fig. 5.3).

5.3.2.2 Smad-Independent Pathways Controlling TGF-β-Mediated Apoptosis

TGF-β can also induce apoptosis through Smad-independent signaling pathways. In this case, to promote cell death, TGF-β signals through the p38 and JNK (c-Jun N-terminal kinase) pathways (Fig. 5.3), both classically associated with responses to cell stress and apoptosis. TGF-β has been known for long time to facilitate activation of JNK (Atfi et al. 1997; Parkinson et al. 2001) and p38 (Hyman et al. 2002; Takekawa et al. 2002; Yoo et al. 2003) in various cell types, but for many years the biochemical links between TGF-β receptors and the actors of the MAPK cascade remained unelucidated.

From a two-hybrid screen, the adaptor protein DAXX has emerged as a target protein interacting physically with TGF-β type II receptor (TβRII) used as bait (Perlman et al. 2001). DAXX is a FAS-receptor-associated protein mediating FAS-induced programmed cell death by activating the JNK pathway. In vitro, DAXX interacts directly with the cytoplasmic domain of TβRII, but not with TβRI. In vivo, it has been found to associate with the TβRI/II receptor complex upon TGF-β stimulation. This association requires the kinase activity of the TβRII receptor, since a kinase-dead mutant of TβRII fails to interact with DAXX (Perlman et al. 2001). DAXX is crucial to TGF-β-induced apoptosis in both B-cell lymphoma cells and mouse hepatocytes, since overexpression of a dominant-negative form of DAXX or artificial *DAXX* silencing inhibits TGF-β-mediated apoptosis in these cells (Perlman et al. 2001). Upon TGF-β stimulation, interestingly, DAXX acts as an adaptor protein facilitating the phosphorylation and subsequent activation of JNK and triggering cell death. DAXX can interact physically with HIPK2 (homeodomain-interacting protein kinase 2), a serine/threonine kinase involved in transcriptional regulation and also apoptosis (Hofmann et al. 2003). The HIPK2-DAXX complex facilitates activation of the JNK protein by the upstream mitogen-activated protein kinase kinases MKK4 and MKK7. Overexpression of both DAXX and HIPK2 leads to potentiation of TGF-β-induced cell death. This effect is mediated by the kinase function of HIPK2, since expression of a kinase-deficient HIPK2 protein fails to increase TGF-β-induced cell death in these cells. Interestingly, HIPK2 also phosphorylates the DAXX protein (Hofmann et al. 2003), but whether this phosphorylation event is required for the adaptor function of DAXX in the specific context of TGF-β-induced apoptosis has not been explored. These findings constitute the

first evidence of a biochemical link between TGF-β receptors and the JNK pathway, leading to the apoptotic machinery (Fig. 5.3).

Concerning activation of the p38 MAPK by TGF-β, previous studies have indicated that TAK1 (TGF-β-activated kinase 1) might be an indirect link between p38 and the TGF-β receptor complex (Shibuya et al. 1996; Yamaguchi et al. 1995). TAK1 is a member of the MAP kinase kinase kinase family, identified as a TβRI-interacting protein (Shibuya et al. 1996; Yamaguchi et al. 1995) almost concomitantly with the discovery of the Smad proteins (Heldin et al. 1997). TAK1 has been proposed to be involved in TGF-β-induced cell death through its ability to activate p38 in a Smad-independent manner (Sano et al. 1999). Until recently, the biochemical link between the TβRI-TAK1 complex and p38 activation remained unidentified (Sorrentino et al. 2008; Yamashita et al. 2008). The E3-ligase TRAF6 can bind constitutively to TβRI. Upon ligand binding and TGF-β receptor TβRI-TβRII hetero-oligomerization, TRAF6 becomes auto-ubiquitinated by polyubiquitin chains linked via their Lys63. TRAF6 then catalyzes Lys63-mediated polyubiquitination of TAK1, and thus activates it. It is important to stress that unlike Lys48-linked polyubiquitination, Lys63-linked polyubiquitination is not a signal targeting proteins for degradation by the proteasome complex. Although its role in the TAK1 activation process is not fully understood, TAK1 polyubiquitination seems to facilitate TAK1 juxtaposition-induced auto-phosphorylation. Alternatively, it might favor a conformational change allowing exposure of the TAK1 catalytic domain. In turn, TAK1 phosphorylates and activates the MAP kinase kinases: MKK3 and MKK6, which ultimately phosphorylate MAPK p38. In this context, Smad7 might act as a docking protein facilitating the phosphorylation cascade, having previously been shown (*1*) to interact with the TAK1, MKK3, and p38 proteins and (*2*) to be required for TGF-β-induced apoptosis in prostate cancer cells (Edlund et al. 2003) (Fig. 5.3).

Interestingly, activation of TAK1 and p38 does not require TβRI kinase activity (Sorrentino et al. 2008; Yamashita et al. 2008), but the presence of TRAF6 and its E3-ubiquitin ligase activity are indispensable to TGF-β-mediated apoptosis in prostate cancer cells (Sorrentino et al. 2008) and hepatocytes (Yamashita et al. 2008). Artificial down-regulation of *TRAF6* in prostate cancer cells or hepatocytes abolishes TGF-β-triggered apoptosis, and similar observations have been made on TRAF6-deficient mouse embryonic fibroblasts (Sorrentino et al. 2008; Yamashita et al. 2008). Moreover, overexpression of a deletion mutant of TRAF6 lacking E3-ubiquitin ligase activity inhibits TGF-β-mediated TAK1 ubiquitination and subsequent activation and also p38 phosphorylation (Sorrentino et al. 2008).

Another adaptor protein has recently been implicated in this noncanonical TRAF6-TAK1 pathway initiated by TGF-β: TTRAP (TRAF and TNF receptor associated protein). TTRAP forms a ternary complex with TAK1 and TRAF6, which can be recruited to TβRI in the presence of the ligand. The presence of TTRAP potentiates the E3-ubiquitin ligase activity of TRAF6, thereby promoting TAK1 ubiquitination. Artificial down-regulation of TTRAP impairs TGF-β-induced activation of p38 (Varady et al. 2011).

5.4 Conclusion

In conclusion, to the extent that the tumor-suppressive mechanisms orchestrated by TGF-β have been deciphered, it is obvious that the cytostatic and apoptotic responses of a cell to TGF-β involve totally different regulatory events and processes. The cytostatic program, on the one hand, involves regulation of a limited number of genes by Smad proteins, with few additional effectors intervening to block the cell cycle before the G1 restriction point. The apoptotic program, on the other hand, involves many modifying enzymes, notably kinases, and also requires Smad-mediated transcriptional regulation in order to favor pro-apoptotic instead of pro-survival pathways. The mechanism by which a cell switches from one program to the other depends on an intricate dialogue with its microenvironment and is still largely unknown.

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Chapter 6
TGF-β as Tumor Suppressor: Lessons from Mouse Models

Ulrich Valcourt, David F. Vincent, and Laurent Bartholin

Abstract Transforming growth factor-β (TGF-β) signals through serine/threonine-kinase receptors to activate several intracellular signaling pathways, including the "canonical" Smad signaling pathway. TGF-β has tumor-suppressive functions through its capacity to induce growth arrest and apoptosis. Escape from the anti-proliferative effects of TGF-β is a hallmark of almost all tumors of epithelial origin. This chapter first depicts the genetic alterations ablating the TGF-β tumor-suppressive functions in human tumors. Then, the chapter presents the genetically engineered mouse models that have been developed by introducing genetic alterations found in humans. These mouse models demonstrated the tumor-suppressive role of TGF-β in vivo shedding light on its intricate relationship with the tumor microenvironment. In all, the presented mouse models of cancer with an impaired TGF-β signaling pathway provide an integrated view of the complex tumor-suppressive role of TGF-β and represent valuable tools for preclinical studies.

Keywords Cre/lox • Digestive tract • Epithelial-to-mesenchymal transition (EMT) • Homotypic cell cannibalism (HoCC) • Mammary gland • Mutations • Pancreas • Smad • Transgenic mice • Xenograft

Abbreviations

ALK5	Activin receptor-like kinase 5
AOM	Azoxymethane
APC	Adenomatous polyposis coli

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BMP	Bone morphogenetic protein
BMPR1A	Bone morphogenetic protein receptor, type IA
CTGF	Connective tissue growth factor
DEN	Diethylnitrosamine
DMBA	7,12-Dimethylbenz(α)anthracene
DPC4	Deleted in pancreatic carcinoma 4
ELF	Embryonic liver fodrin
EMT	Epithelial-to-mesenchymal transition
FAP	Familial adenomatous polyposis
HoCC	Homotypic cell cannibalism
IPMN	Intraductal papillary mucinous neoplasms
KSSTT	Pdx1-Cre; $KRAS^{G12D}$; Smad4-$KO^{L/L}$; TIF1γ-$KO^{L/L}$
LOH	Loss of heterozygosity
LRP	Low-density lipoprotein receptor-related protein
LSL	Lox-stop-lox
MAPK	Mitogen-activated protein kinase
MCN	Mucinous cystic neoplasms
Min	Multiple intestinal neoplasia
MMP	Matrix metalloproteinase
MMTV	Mouse mammary tumor virus
MSI	Microsatellite instability
PanIN	Pancreatic intraepithelial neoplasms
PDAC	Pancreatic ductal adenocarcinoma
PDGF	Platelet-derived growth factor
PyVmT	Polyoma virus middle T antigen
SCC	Squamous cell carcinoma
TGF-β	Transforming growth factor β
TGF-α	Transforming growth factor α
TIF1	Transcriptional intermediary factor 1
TPA	12-O-tetradecanoylphorbol-13-acetate
TβRI	TGF-β type I receptor
TβRII	TGF-β type II receptor
WAP	Whey acidic protein

6.1 Introduction: TGF-B Signaling and Genetic Alterations in Human Tumors

Transforming growth factor-β (TGF-β) is a secreted polypeptide belonging to a large family of cytokines and growth factors including TGF-βs, bone morphogenetic proteins (BMPs), and activins. The three isoforms of TGF-β (TGF-β1, TGF-β2, and TGF-β3) are secreted to different levels by most cell types, and they act as positive and negative regulators of differentiation and proliferative programs

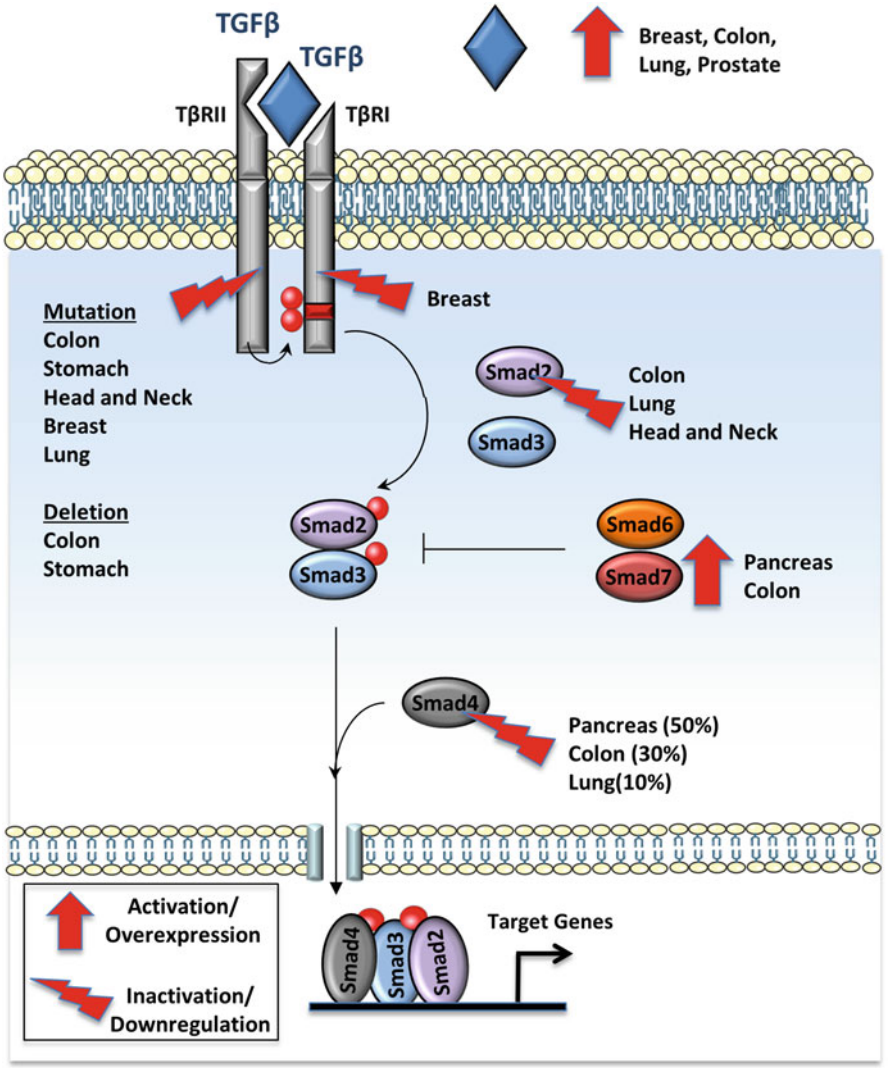

Fig. 6.1 Genetic alterations in human tumors affecting TGF-β signaling

(Letterio and Roberts 1998). TGF-βs play a crucial role in physiological processes involved in embryonic development, immune responses, cell growth control, and wound healing. All three TGF-β isoforms signal through a common heterotetrameric serine/threonine-kinase receptor complex (Massague 1992) comprising TβRII and TβRI (also termed activin receptor-like kinase 5, ALK5) (Fig. 6.1). After binding to its receptors, TGF-β induces phosphorylation of TβRI, which phosphorylates receptor-associated Smad2 and Smad3 (R-Smads). Phosphorylated Smad2 and

Smad3 interact with Smad4. The Smad2, -3, -4 complex accumulates within the nucleus, binds to DNA, and regulates transcription of target genes. In addition to this "canonical" Smad signaling pathway, TGF-β activates Smad-independent pathways (MAPK, RHOA, PI3K/AKT) (Mu et al. 2012).

For a comprehensive review of the signaling events and molecular mechanisms by which TGF-β orchestrated cell growth arrest and apoptosis, the reader is invited to read Chap. 5. Escape from the anti-proliferative effects of TGF-β signaling is an obligate step for almost all tumors of epithelial origin. For this, cancer cells have positively selected inactivating mutations affecting genes coding for key players involved in TGF-β signaling, such as the *TGF-β* receptor genes and *Smad* genes. These mutations render cancer cells insensitive to the growth-inhibiting action of TGF-β. Here we shall focus on those mutations which have provided the information most relevant to better understanding the tumor-suppressive role of TGF-β (mainly Smad4, TβRII, and Smad2 mutations) (Fig. 6.1). For a more complete list of mutations affecting TGF-β signaling in human tumors, the reader is invited to consult comprehensive reviews published by others (Kim et al. 2000; Levy and Hill 2006; Massague 2008).

Germline mutations in *Smad4* or *BMPRIA* (bone morphogenetic protein receptor, type IA) are found in over 50 % of cases of juvenile polyposis syndrome (JPS) (Aretz et al. 2007; Howe et al. 1998; van Hattem et al. 2008). JPS patients develop during childhood or adolescence multiple polyps in the gastrointestinal tract, associated with a high risk of colorectal cancer (50 % by the age of 60 years). Beyond these germline mutations concerning a minority of cancer patients, somatic mutations compromising the TGF-β signaling pathway are recurrently found in many sporadic tumors (Fig. 6.1). For instance, the locus 18q21.1 containing *Smad4* is deleted in over 50 % of human pancreatic cancers (this locus is also known as *DPC4, Deleted in Pancreatic Carcinoma 4*) (Hahn et al. 1996). Mutation of *Smad4* appears, indeed, as a preferential means of tumor escape from the tumor-suppressive functions of TGF-β signaling, since Smad3 is practically never mutated in human neoplasms and Smad2 mutations are rather rare. Why this is so remains unclear, but it has been hypothesized that Smad4, being involved in the signaling of all the TGF-β superfamily members, might additionally inactivate BMP signaling, which is also involved in tumor suppression. This hypothesis is supported by the presence of BMPRIA mutations in a subset of human JPS patients (Howe et al. 2001). Alternatively, Smad2 and Smad3 might be relatively spared by genetic alterations as compared to Smad4 because of an “R-Smad dependency” of many tumors. It is tempting to speculate that retention of Smad2 and Smad3 might confer a selective advantage to the tumor by driving invasive programs (epithelial-to-mesenchymal transition - EMT, immunosuppression, extracellular remodeling). For instance, Smad2 and Smad3 still accumulate in the nucleus in Smad4-deficient human pancreatic cancer cells (Subramanian et al. 2004) suggesting that R-associated Smad proteins have nuclear functions independently of Smad4. This hypothesis is comforted by the Smad4-independent association of Smad2 and Smad3 with inhibitor of nuclear factor κ-B kinase subunit alpha (IKKα) (Descargues et al. 2008) or transcriptional intermediary factor 1 γ (TIF1γ) (He et al. 2006).

TβRII is rarely altered in pancreatic cancers but is deleted in the majority of colorectal cancers associated with microsatellite instability (MSI) (Markowitz et al. 1995). Why is it that in MSI colon cancer mutations occur more frequently in TβRII than in any other gene involved in TGF-β signaling? MSI colon cancers are characterized by defective DNA mismatch repair (MMR), so the tumors accumulate mutations in microsatellite sequences. The TβRII gene contains "microsatellite-like" sequences rendering it particularly vulnerable to mutations in cases of MSI colorectal cancer. Interestingly, TβRII mutations resulting from MSI are positively selected, enabling the tumor to escape the growth-inhibiting effect of TGF-β (Biswas et al. 2008). This is a genuine example of the capacity of human neoplasms to escape the tumor-suppressive effect of TGF-β thanks to preexisting intrinsic tumor properties (microsatellite instability, in the case of MSI colorectal tumors).

Genome-scale exon sequencing has confirmed that TGF-β signaling mutations in the genes encoding the TβRII and Smad proteins are a hallmark of certain types of cancer, as illustrated by the sequencing of 24 pancreatic tumors (Jones et al. 2008) and 276 colorectal cancers (Cancer Genome Atlas Network 2012). In contrast, TGF-β mutations failed to emerge as a tumor signature from a study on 92 medulloblastomas (Pugh et al. 2012) and 100 breast tumors (Stephens et al. 2012). This raises the question: how do cancer cells having no recurrent genomic alteration that compromises TGF-β signaling escape TGF-β-mediated growth inhibition? One should consider, firstly, that some tumors derive from cells that are not sensitive to TGF-β growth inhibition in the first place. This is the case of sarcomas, which derive from mesenchymal cells intrinsically insensitive to the cytostatic action of TGF-β and which therefore do not require impairment of the TGF-β signaling pathway in order to develop. Secondly, tumors displaying a low rate of mutation in genes involved in TGF-β signaling may have escaped the tumor-suppressive effect of TGF-β by other means. For instance, repression of TGF-β receptor gene transcription by DNA methylation has been reported in a human gastric cancer (Kang et al. 1999) and human breast tumors (Hinshelwood et al. 2007). Also reported in human tumors is up-regulation of negative regulators of TGF-β signaling. For instance, SKI/SNON expression is increased in breast cancer (Zhang et al. 2003), colorectal cancer (Buess et al. 2004), melanoma (Medrano 2003; Poser et al. 2005; Reed et al. 2001), and esophageal squamous cell carcinoma (Fukuchi et al. 2004).

6.2 Mouse Models of TGF-β-Induced Tumor Suppression

Many mouse models have been developed to decipher the role of TGF-β in cancer. They rely on introducing into the murine genome the human mutations described in paragraph 1 with the ultimate goal to compromise in vivo the tumor-suppressive functions described in paragraph 2.

Homozygous TGF-β1^{null} mice develop autoimmune disorders, culminating in their death by 3 weeks of age (Kulkarni et al. 1993; Shull et al. 1992). Half of the TGF-β1^{null} embryos die in utero because of hematopoiesis and vasculogenesis

Table 6.1 Mouse models with whole-body homozygous inactivation of central regulators of the TGF-β signaling

Knockout gene	References	Phenotype
TGF-β1^{null}	Shull et al. (1992)	Multifocal inflammatory disease, death before 1 month
	Kulkarni et al. (1993)	Multifocal inflammatory disease, death before 1 month
	Dickson et al. (1995)	Multifocal inflammatory disease, death before 1 month, 50 % of death at E10.5 because of defective hematopoiesis and vasculogenesis
TGF-β2^{null}	Sanford et al. (1997)	Wide range of developmental defects causing perinatal death (cardiac, lung, craniofacial, limb, spinal column, eye, inner ear, and urogenital defects)
TGF-β3^{null}	Proetzel et al. (1995)	Failure of the palatal shelves to fuse leading to cleft palate
TβRI/ALK5^{null}	Larsson et al. (2001)	Death at E10.5 because of defective hematopoiesis and vasculogenesis
TβRII^{null}	Oshima et al. (1996)	Death at E10.5 because of defective hematopoiesis and vasculogenesis
TβRIII^{null}/Betaglycan	Stenvers et al. (2003)	Embryonic lethality caused by defects in heart and liver
Smad2^{null}	Nomura and Li (1998) Waldrip et al. (1998) Weinstein et al. (1998)	Embryonic death because gastrulation defect
Smad3^{null}	Zhu et al. (1998)	Colorectal cancers
Smad4^{null}	Yang et al. (1998)	Death at E8.5, gastrulation defect
	Sirard et al. (1998)	Death at E8.5, gastrulation defect
	Takaku et al. (1998)	Death at E8.5, gastrulation defect

defects in the yolk sac (Dickson et al. 1995). TGF-β2^{null} (Sanford et al. 1997) and TGF-β3^{null} (Proetzel et al. 1995) mice are viable, but display multiple developmental defects. Homozygous knockouts of TβRI/ALK5 (Larsson et al. 2001) or TβRII (Oshima et al. 1996) are embryonically lethal. Knockout TβRIII (Betaglycan) mice develop embryonic lethal proliferative defects in heart and apoptosis in liver (Stenvers et al. 2003). Smad3^{null} mice spontaneously develop colorectal cancers (Zhu et al. 1998). Inactivation either of the other two Smad proteins involved in TGF-β signaling, Smad2 (Nomura and Li 1998; Waldrip et al. 1998; Weinstein et al. 1998) and Smad4 (Sirard et al. 1998; Takaku et al. 1998; Yang et al. 1998), leads to embryonic death (Table 6.1).

Altogether, these mouse models provide a perfect illustration of the pleiotropic effects of TGF-β and of its crucial role in development and homeostasis, as all these whole-organism mutations lead to embryonic or perinatal lethality (with the exception of Smad3 inactivation, responsible for colorectal tumors). As the severity of the induced phenotypes limits the utility of these models in cancer studies, several strategies have been devised to get around this limitation. In this paragraph, we will describe in detail informative mouse models that have been obtained by targeting

the mammary gland, digestive tract, exocrine pancreas. Mouse models of skin cancer, which constitute appropriate systems for exploring the effects of external carcinogens such as chemicals and ultraviolet irradiation, will be described in Chap. 9. Skin models along with models targeting other organs [e.g., the liver (Tang et al. 1998), prostate (Kundu et al. 2000; Pu et al. 2009), or head and neck (Bian et al. 2009; Bornstein et al. 2009)] are listed in Table 6.2 but are not extensively described in the text.

Table 6.2 Mouse models demonstrating the tumor-suppressive role of TGF-β

Organ	Genetic alteration	Organ specificity	Oncogenic cooperation	References
Breast	TGF-β1$^{S223/225}$	Yes	Alone	Pierce et al. (1993)
	TGF-β1$^{S223/225}$	Yes	Alone	Jhappan et al. (1993)
	TGF-β1$^{S223/225}$	Yes	Alone	Kordon et al. (1995)
	TGF-β1$^{S223/225}$	Yes	TGF-α	Pierce et al. (1995)
	TGF-β1$^{S223/225}$	Yes	Alone	Buggiano et al. (2001)
	TGF-β1$^{S223/225}$	Yes	Alone	Boulanger and Smith (2001)
	TGF-β1$^{S223/225}$	Yes	Neu	Muraoka et al. (2003)
	TGF-β1$^{S223/225}$	Yes	Alone	Boulanger et al. (2005)
	TGF-β3-KO	Whole body	Alone	Nguyen and Pollard (2000)
	TβRI^{CA}	Yes	PyVmT	Muraoka-Cook et al. (2004)
	TβRI^{CA}	Yes	Neu	Muraoka-Cook et al. (2006)
	TβRII^{DN}/TβRI^{CA}	Yes	Neu	Siegel et al. (2003)
	TβRII^{DN}	Yes	DMBA	Bottinger et al. (1997a)
	TβRII^{DN}	Yes	Alone	Gorska et al. (1998)
	TβRII^{DN}	Yes	None and TGF-α	Gorska et al. (2003)
	TβRII-KO^{het}	Yes	PyVmT	Fang et al. (2011)
	TβRII-KO^{null}	Yes	PyVmT	Forrester et al. (2005)
	TβRII-KO^{null}	Yes	Alone	Cheng et al. (2005)
	TβRII-KO^{null}	Yes	Alone	Yang et al. (2008)
	TβRII-KO^{null}	Yes	PyVmT	Bierie et al. (2008)
	TβRII-KO^{null}	Yes	PyVmT	Bierie et al. (2009)
	None (treatment with TβRII blocking antibody)	N/A	PyVmT	Muraoka et al. (2002)
	Smad4-KO^{null}	Yes	Alone	Li et al. (2003)
	Smad3-KO^{null}	Whole body	Alone	Yang et al. (2002)
Intestine	TGF-β1^{null}	Whole body	Rag2^{null}	Engle et al. (2002)
	TGF-β1^{null}	Whole body	Rag2^{null}	Engle et al. (1999)
	TβRI-KO^{het}	Whole body	Apc^{Min}	Zeng et al. (2009)

(continued)

Table 6.2 (continued)

Organ	Genetic alteration	Organ specificity	Oncogenic cooperation	References
	TβRII-KO^{null}	Yes	Azoxymethane (AOM)^{null}	Biswas et al. (2004)
	TβRII-KO^{null}	Yes	Apc^{1638N}	Munoz et al. (2006)
	TβRII-KO^{null}	Yes	KRAS^{G12D} and Apc^{1638N}	Trobridge et al. (2009)
	Smad2-KO^{het}	Whole body	ApcΔ^{716}	Takaku et al. (2002)
	Smad2-KO^{het}	Whole body	Apc^{580D}	Hamamoto et al. (2002)
	Smad3-KO^{null}	Whole body	Alone	Zhu et al. (1998)
	Smad3-KO^{null}	Whole body	Helicobacter spp.	Maggio-Price et al. (2006)
	Smad3-KO^{null}	Whole body	ApcMin	Sodir et al. (2006)
	Smad4-KO^{het}	Whole body	ApcΔ^{716}	Takaku et al. (1998)
	Smad4-KO^{het}	Whole body	Alone	Takaku et al. (1999)
	Smad4-KO^{het}	Whole body	Alone	Xu et al. (2000)
	Smad4-KO^{het}	Whole body	Alone	Hohenstein et al. (2003)
	Smad4-KO^{hetE6D}	Whole body	Apc^{1638N}	Alberici et al. (2006)
	Smad4-KO^{het}	Whole body	Elf^{het}	Redman et al. (2005)
	Smad4-KO^{het}	Whole body	Elf^{het}	Tang et al. (2005)
	Smad4-KO^{het}	Whole body	Elf^{het}	Katuri et al. (2006)
	TβRII^{fspKO}	Mesenchymal compartment	Alone	Bhowmick et al. (2004)
	Smad4-KO^{het}	Whole body	ApcΔ^{716}	Kitamura et al. (2007)
	Smad4-KO^{het}	Yes	Apc^{1638N}	Freeman et al. (2012)
	Smad4-KO^{het}	Yes	Alone	Kim et al. (2006)
Skin	TGF-β1$^{S223/225}$	Yes	Alone	Sellheyer et al. (1993)
	TGF-β1$^{S223/225}$	Yes	TPA	Cui et al. (1995)
	TGF-β1$^{S223/225}$	Yes	TPA	Fowlis et al. (1996)
	TGF-β1$^{S223/225}$	Yes	TPA	Cui et al. (1996)
	TGF-β1$^{S223/225}$	Yes	DMBA	Weeks et al. (2001)
	TGF-β1$^{S223/225}$	Yes	Alone	Liu et al. (2001)
	TGF-β1$^{S223/225}$	Yes	Alone	Wang et al. (1999)
	TβRII^{DN}	Yes	Alone	Wang et al. (1997)
	TβRII^{DN}	Yes	Alone	Amendt et al. (1998)
	TβRII^{DN}	Yes	TPA	Go et al. (1999)
	TβRII^{DN}	Yes	TPA,DMBA	Go et al. (2000)
	TβRII^{DN}	Yes	Alone	Amendt et al. (2002)
	TβRII^{DN}+ TβRII-KO^{null}	Yes	DMBA/TPA	Han et al. (2005)
	TβRII-KO^{null}	Yes	Ha-Ras^{V12}	Guasch et al. (2007)
	Smad3-KO^{null}	Whole body	Alone	Ashcroft et al. (1999)
	Smad4-KO^{het}	Whole body	Elf^{het}	Redman et al. (2005)
	Smad4-KO^{null}	Yes	Alone	Li et al. (2003)

(continued)

Table 6.2 (continued)

Organ	Genetic alteration	Organ specificity	Oncogenic cooperation	References
	Smad4-KO^{null}	Yes	PTEN $^{-/-}$	Yang et al. (2005)
	Smad4-KO^{null}	Yes	Alone	Qiao et al. (2006)
Pancreas	TβRII^{DN}	Whole body	Alone	Bottinger et al. (1997b)
	TβRII-KO^{null}	Yes	K-Ras^{G12D}	Ijichi et al. (2006)
	TβRII-KO^{null}	Yes	K-Ras^{G12D}	Ijichi et al. (2011)
	Smad4-KO^{null}	Yes	K-Ras^{G12D}	Bardeesy et al. (2006)
	Smad4-KO^{null}	Yes	K-Ras^{G12D}	Kojima et al. (2007)
	Smad4-KO^{null}	Yes	K-Ras^{G12D}	Izeradjene et al. (2007)
	Smad7^{Tg}	Yes	Alone	Kuang et al. (2006)
	TIF1γ-KO^{null}	Yes	K-Ras^{G12D}	Vincent et al. (2009)
	TIF1γ-KO^{null}	Yes	K-Ras^{G12D}	Vincent et al. (2012)
	NupR1-KO^{null}	Whole body	K-Ras^{G12D}	Cano et al. (2012)
Head and Neck	TβRI-KO^{null}	Yes	DMBA	Bian et al. (2009)
	Smad4-KO^{null}	Yes	Alone	Bornstein et al. (2009)
Prostate	TβRII^{DN}	Yes	SV40-Large T	Pu et al. (2009)
	TβRII^{fspKO}	Mesenchymal compartment	none	Bhowmick et al. (2004)
	TβRII^{DN}	Yes	none	Kundu et al. (2000)
Liver	TGF-β1^{het}	Whole body	Diethylnitro-samine (DEN)	Tang et al. (1998)
	Smad3^{Tg}	Yes	Diethylnitro-samine (DEN)	Yang et al. (2006)

6.2.1 The Mammary Gland

In mice, mammary gland development (Sternlicht 2006) begins shortly after mid-gestation (E10.5). It is characterized by the presence at birth of a rudimentary system of branching ducts opening into the nipple. From birth to puberty, there is but little development of the branching duct network. At puberty (~5 weeks of age), a massive wave of branching occurs, so that the ducts fill the entire mammary fat pad of the young adult. During the estrus cycles, the mammary gland undergoes successive cycles of proliferation and regression. Pregnancy is associated with massive cell proliferation in the mammary gland, along with lobular-alveolar differentiation to produce milk. Lactation in the mother's mammary gland starts when the pups are born. After weaning, lactation stops and the secreting lobular-alveolar network of the mammary gland involutes, an event resulting from massive apoptosis.

In the TGF-β1$^{S223/225}$ mutant, cysteine residues at positions 223 and 225 are replaced with serine residues so that TGF-β1 is secreted in an active form (this mutant is unable to bind the latency-associated protein, LAP). Transgenic mice expressing TGF-β1$^{S223/225}$ have been generated, with expression of the TGF-β1$^{S223/225}$ transgene under the control of either the promoter of the mouse mammary tumor

virus (MMTV) gene (associated with early differentiation of the mammary gland) or the promoter of the whey acidic protein (WAP) gene (associated with late pregnancy, lactation, and early involution). In MMTV-TGF-β1$^{S223/225}$ mice (Pierce et al. 1993), duct development is severely compromised, this leading to mammary gland hypoplasia. WAP-TGF-β1$^{S223/225}$ mice develop normal ducts but are not capable to support lactation as they present a severe deficiency in their ability to form secretory lobules during pregnancy (Jhappan et al. 1993) as a consequence of the early senescence of the regenerative capacity of the mammary ductal epithelium (Kordon et al. 1995). TGF-β3 expression from the β-lactoglobulin promoter in the lactating epithelium of mice is likewise reported to induce involution of the mouse mammary gland (Nguyen and Pollard 2000). Models have also been developed to inhibit TGF-β signaling in the mammary gland. As expected, these models give rise to phenotypes opposite to those just described. Thus, virgin MMTV-TβRII^{DN} (dominant negative mutant form of TβRII) (Bottinger et al. 1997b; Gorska et al. 1998) and MMTV-Cre/TβRII-KO$^{L/L}$ (a conditional TβRII-knockout allele) (Forrester et al. 2005) mice display hyperplasia and differentiation of the mammary glands. The MMTV-TβRII^{DN} model shows delayed involution of the mammary gland after forced weaning of pups during lactation (Gorska et al. 2003).

Smad3^{null} mice, which develop colorectal tumors (Zhu et al. 1998), also show under-developed mammary glands and involution defects (Yang et al. 2002). The mammary glands of WAP-Cre/TβRII-KO$^{L/L}$ mice likewise fail to undergo complete involution of the lobular-alveolar secreting structures (Bierie et al. 2009). Defective mammary gland involution is due to compromised apoptosis, a crucial biological process controlled by TGF-β. Unexpectedly, MMTV-Cre/Smad4$^{L/L}$ and WAP-Cre/Smad4$^{L/L}$ mice display no involution defects or obvious developmental defects (Li et al. 2003), but they do show spontaneous mammary tumor formation (Li et al. 2003). The mammary glands of MMTV-Cre/Smad4-KO$^{L/L}$ mice show increased proliferation, alveolar hyperplasia, and trans-differentiation into squamous epithelial cells, and they finally develop squamous cell carcinoma (SCC). To date, this is the only model of spontaneous mammary tumor formation in genetically engineered mice with compromised TGF-β signaling. The development of mammary tumors when Smad4 is knocked down may be due to more efficient inactivation of TGF-β signaling than in TβRII^{DN} mice (Bottinger et al. 1997b; Gorska et al. 1998). Alternatively, it might be due to inactivation of a non-TGF-β pathway such as the BMP pathway, which should occur upon Smad4 knockdown but not in the TβRII-KO model (Forrester et al. 2005).

Altogether, these observations demonstrate that TGF-β exerts anti-proliferative effects constraining mammary duct development in virgin mice and enabling involution of the mammary gland at the end of lactation. They also functionally validate the pivotal role of Smad4 and TβRII in limiting cell proliferation and tumor formation, in line with the high prevalence of genetic alterations affecting both genes in human neoplasms.

Because disruption of TGF-β signaling is not sufficient to induce mammary tumors in most of the models described above (only Smad4 inactivation is sufficient to induce mammary tumors), mutations affecting TGF-β signaling have been

combined with various oncogenic factors. This has yielded robust models of predisposition to mammary tumors. These approaches include:

1. Exploring the effects of chemical carcinogens in mice with attenuated TGF-β signaling. It appears that MMTV-Cre/TβRII^{DN} mice are more sensitive to 7,12-dimethylbenz(α)anthracene (DMBA)-induced mammary tumorigenesis than control littermates (Bottinger et al. 1997b). This sensitization indirectly confirms the tumor-suppressive function of TGF-β.
2. Studying the role of TGF-β in relation to mammary tumorigenesis in multiparous females. MMTV-Cre/TβRII^{DN} mice having experienced two pregnancies with partial involution of the secreting lobular-alveolar structures have an increased risk of mammary tumor formation (Gorska et al. 2003). This observation suggests that TGF-β is a potent inhibitor of the transformation of hyperplastic tissues, known to be more likely to evolve towards neoplastic lesions. This observation identifies TGF-β-induced apoptosis, responsible for mammary gland involution after lactation, as a crucial tumor-suppressive mechanism.
3. Assessing susceptibility to the development of mammary tumors after MMTV infection. Milk-transmitted MMTV infection has been extensively used to induce mammary tumors [for reviews, see (Callahan and Smith 2000; Ross 2010)]. This line of research has yielded contradictory results: one group found WAP-TGF-β1$^{S223/225}$ mice to show no change in susceptibility to MMTV infection or subsequent tumor development, despite a severe decrease in lobular alveolar development (Buggiano et al. 2001); another group found WAP-TGF-β1$^{S223/225}$ mice to display a decreased risk of mammary cancer after MMTV infection (Boulanger and Smith 2001), thanks to early senescence in the mammary stem cell population (Boulanger et al. 2005). Although the reason for this discrepancy is unclear, it is important to note that TGF-β may prevent mammary tumors by inducing the senescence of stem cells, in addition to its pro-apoptotic effect on differentiated cells.
4. Exploiting oncogene-induced mouse models of mammary cancer. Three oncogenes are classically used in transgenic mice: transforming growth factor alpha (TGF-α), Her-2/neu (erbB-2), and polyoma virus middle T antigen (PyVmT). MMTV-TGF-β1$^{S223/225}$/MMTV-TGF-α mice develop significantly fewer mammary tumors than MMTV-TGF-α mice (Pierce et al. 1995). Conversely, MMTV-Cre/TβRII-KO$^{L/L}$/MMTV-PyVmT mice develop tumors more quickly than MMTV-PyVmT mice (Forrester et al. 2005), and MMTV-TβRII^{DN}/MMTV-TGF-α develop mammary tumors at a higher frequency than MMTV-TGF-α mice (Gorska et al. 2003). WAP-Cre/TβRII-KO$^{L/L}$/MMTV-PyVmT mice also present an accelerated mammary tumorigenesis (Bierie et al. 2008). Finally, MMTV-TβRI^{CA}/MMTV-Neu mice develop primary tumors later (Siegel et al. 2003) and MMTV-TβRIIDN/MMTV-Neu mice develop them earlier than MMTV-Neu mice (Siegel et al. 2003). Together, these results indicate that TGF-β efficiently opposes TGF-α-, PyVmT-, and Her2/Neu- oncogene-driven transformation of the epithelial mammary tissue, illustrating once again its tumor-suppressor role.

5. Disrupting TGF-β signaling in the stromal compartment instead of the epithelial compartment. This approach relies on a mouse model in which TGF-β signaling is specifically abolished in the mesenchymal compartment and involves associating a Cre-recombinase controlled by the FSP1 (fibroblast specific protein-1; S100A4) promoter with a conditional TβRII-knockout allele in FSP1-Cre/TβRII$^{L/L}$ compound transgenic mice. These mice develop pre-neoplastic lesions resulting in prostate and stomach carcinomas and display severely compromised development of the mammary gland (Bhowmick et al. 2004). Furthermore, it appears that TβRII-deficient fibroblasts can facilitate tumor growth when grafted with PyVmT or 4T1 mammary carcinoma cells (Cheng et al. 2005). This finding has been further validated in MMTV-PyVmT/FSP1-Cre/TβRII$^{L/+}$ mice developing autochthonous mammary tumors (Fang et al. 2011). Finally, xenografts onto the chicken embryo chorioallantoic membrane revealed that the absence of TβRII in fibroblasts could promote invasiveness of murine mammary carcinoma cells (Matise et al. 2012). Altogether, these studies clearly demonstrate that the tumor-suppressive function of TGF-β relies also on microenvironmental effects.

Importantly, although this point lies outside the scope of this chapter, the mammary gland is particularly suitable for investigating the dual role of TGF-β during carcinogenesis, since many studies based on mouse models of mammary tumors indicate that once transformation has occurred and a tumor has become established, TGF-β actually facilitates tumor progression and subsequent metastatic dissemination, in contrast to its tumor-suppressive effect on the normal and hyperplastic mammary gland (Bottinger et al. 1997a; Forrester et al. 2005; Gorska et al. 2003; Muraoka-Cook et al. 2004; Muraoka-Cook et al. 2006; Muraoka et al. 2002; Muraoka et al. 2003). Tumor-promoter functions of TGF-β are developed in Chap. 7.

6.2.2 The Digestive Tract

The digestive tract is anatomically divided into different parts: the mouth, esophagus, stomach, small intestine, colon, and rectum. The intestinal lumen is lined with a specialized epithelium organized into crypts and villi (van der Flier and Clevers 2009). Stem and proliferating cells are located in the crypts, whereas differentiated cells (enterocytes, entero-endocrine cells, and goblet cells) are located in the villi. Paneth cells constitute the only differentiated cell population settling in the crypts (they do not migrate "upward"). Colorectal adenocarcinoma is the most frequent tumor of the digestive tract. In 1990, Fearon and Vogelstein proposed a model according to which this cancer arises through an ordered sequence of mutations called the adenomacarcinoma sequence (Fearon and Vogelstein 1990). As mentioned earlier in this chapter, the spontaneous development of colorectal tumors in Smad3^{null} mice constitutes strong evidence of the tumor-suppressive role of TGF-β

(Zhu et al. 1998). To explore more precisely the role of TGF-β signaling in cancers of the digestive tract, different cancer predisposition models have been developed:

1. Confirmation of the tumor-suppressive effect of TGF-β signaling on digestive tract tumorigenesis was first obtained in heterozygous Smad4-KO^{het} mice. These mice develop polyps in the proximal gastrointestinal tract, confirming the central role of Smad4 in tumor suppression (Takaku et al. 1999; Xu et al. 2000). Smad4-KOE6sad (sad, serrated adenomas) is a null mutant allele caused by a spontaneous deletion in a splice acceptor site, sufficient to induce serrated adenomas and mixed polyposis (Alberici et al. 2006). Interestingly, knocking down Smad4 in T-lymphocytes (CD4-Cre/Smad4-KO$^{L/L}$) results in epithelial tumors of the oral cavity, stomach, duodenum, rectum, and colon (Kim et al. 2006). This recent observation constitutes new evidence that TGF-β signaling in the microenvironment plays an active role in constraining epithelial tumorigenesis.
2. The best-represented category of digestive tract cancer predisposition models relies on associating TGF-β signaling mutations with mutations that inactivate the adenomatous polyposis coli (APC) gene, which encode an inhibitor of the WNT/β-catenin signaling pathway (van der Flier and Clevers 2009). This pathway is particularly important in the digestive tract, as it maintains the crypt as a "proliferative, undifferentiated, and multipotent" compartment. In contrast, the WNT/β-catenin is not active in the villi, which constitute the "differentiated" compartment. In the absence of WNT ligands, β-catenin binds to a complex containing GSK3β (glycogen synthase kinase-3β), CK1 (casein kinase 1), axin (conductin), and APC. This complex phosphorylates β-catenin, targeting it for ubiquitination and degradation. In the presence of WNT ligands, the receptors Frizzled and LRP (low-density lipoprotein receptor-related protein) are activated, leading to inhibition of the "destruction complex" and stabilization of β-catenin, which can accumulate inside the nucleus and eventually activate transcription of growth-promoting genes. The canonical WNT signal transduction pathway is frequently impaired in human cancers, especially those arising in the digestive tract. As the most common alterations of the WNT/β-catenin pathway in cancer are APC-inactivating and β-catenin-activating mutations, several mouse models have been developed with APC mutations. The different mutants develop small intestinal polyps that are histologically indistinguishable, but the average number of polyps is different according to the mutation: 3 in APC^{1638N} mutants, 30 in APC^{Min} (Min, multiple intestinal neoplasia) mutants, and 300 in APCΔ^{716} mutants. These mouse models are somewhat evocative of human familial adenomatous polyposis (FAP, caused by deletions on chromosome 5q encompassing the APC gene), but in contrast to mice with APC mutations, human FAP patients, who present an increased risk of colorectal cancer, develop polyps mainly in the colon and rectum (not in the small intestine).

   Because TGF-β is impaired in colorectal cancers as a result of mutations affecting the genes encoding the TGF-β receptors and Smad proteins, these

mutations have been combined with APC mutations in transgenic mouse models. Whereas mice homozygous for a Smad3-inactivating mutation spontaneously develop colorectal cancers between 18 and 24 weeks of age (Zhu et al. 1998), APC^{min}/$Smad3^{null}$ mice develop tumors much earlier and are moribund by the age of 8 weeks (Sodir et al. 2006). $APC\Delta^{716}$/Smad4-$KO\Delta^{Ex1/+}$ mice develop colorectal cancers (Takaku et al. 1998) and permitted the identification of a new type of immature myeloid cell (iMC), expressing matrix metalloproteinases (MMP9 and MMP2) and the CC-chemokine receptor 1 (CCR1), present at the invasion front toward CCL9 (the CCR1 ligand), to facilitate tumor invasion (Kitamura et al. 2007). Inactivation of CCR1 in $APC\Delta^{716}$/Smad4-$KO\Delta^{Ex1/+}$/CCR1-KO^{null} compound mice inhibits the accumulation of iMCs preventing the tumor invasion. Whereas Smad4-$KO^{E6sad/+}$ (Hohenstein et al. 2003) develop serrated adenomas and mixed polyposis, APC^{1638N}/Smad4-$KO^{E6sad/+}$ mice have been shown to develop aggressive colorectal cancers (Alberici et al. 2006). As observed in human tumors, the onset of colorectal tumors in mouse models is systematically associated with Smad4 loss of heterozygosity (LOH), indicating that total loss of Smad4 function is required for transformation, rather than haploid insufficiency. Heterozygous inactivation of Smad2 in a mutated APC background was shown in one study to accelerate tumor progression (Hamamoto et al. 2002) and in another study to have no effect (Takaku et al. 2002). The cause of this discrepancy remains unclear but may be due to the use of different mutant APC alleles. Heterozygous inactivation of TβRI in association with the APC^{Min} mutation is associated with a twofold increase in the number of intestinal tumors and with colorectal cancer onset without loss of heterozygosity at the TβRI locus (Zeng et al. 2009). Thus, in contrast to what is observed upon Smad4 inactivation, haploid insufficiency of TβRI is sufficient to facilitate tumorigenesis. Finally, Villin-Cre-dependent activation of the conditional TβRII-$KO^{L/L}$ conditional allele is associated with colorectal cancer development in an APC^{1638N} background (Munoz et al. 2006). The molecular interaction between the TGF-β signaling and the WNT/β-catenin signaling has been recently described by performing microarray performed with biological material prepared from adenomas from $APC\Delta^{1638/+}$/K19-Cre-ERT2/$Smad4^{L/L}$, which revealed increased levels of β-catenin mRNA and WNT signaling (Freeman et al. 2012).

Altogether, these models show that TGF-β-pathway-inactivating mutations strongly potentiate the capacity of APC mutants to induce colorectal cancers. These mouse models clearly demonstrate that progression of colorectal cancers involves inactivation of TGF-β signaling illustrating its tumor suppressor effect. Notably, by combining APC mutations with TGF-β signaling mutations, it is possible to better mimic human FAP, as the lesions appear in the distal intestine.

3. A third category of digestive tract cancer predisposition models relies on the association of TGF-β signaling mutations with mutations affecting genes other than APC. The ELF protein (embryonic liver fodrin), identified in foregut progenitors, belongs to the β-spectrin family involved in cell polarity (Machnicka

et al. 2012). Double heterozygous Smad4-KO^{het}/ELF-KO^{het} mutants develop colorectal cancers and also gastric and liver tumors (Katuri et al. 2006; Redman et al. 2005; Tang et al. 2005). Activating KRAS mutations are classically associated with the transition from the adenoma to the carcinoma state. LSL-KRAS^{G12D} (LSL, Lox-Stop-Lox) is a conditional (Cre-inducible) allele encoding a constitutively active KRAS mutant (KRAS^{G12D}). Villin-Cre/LSL-KRAS^{G12D}/TβRII-KO$^{L/L}$ mice develop small intestinal and colorectal carcinomas by the age of 20 weeks (Trobridge et al. 2009).

4. A fourth strategy for producing digestive tract cancer predisposition models involves mice with different genetic backgrounds to avoid the lethal inflammatory phenotype observed in mice with compromised TGF-β signaling due to TGF-β or Smad inactivation. To avoid the generalized inflammatory disorders that cause early death of TGF-β1^{null} mice at ~3 weeks of age (Kulkarni et al. 1993; Shull et al. 1992), homozygous inactivation of TGF-β1 was introduced into immunodeficient RAG2^{null} mice, lacking both B and T cells. TGF-β1^{null}/RAG2^{null} double-mutant mice do not develop inflammatory disease, live to adulthood, and develop colorectal cancers by the age of 5 months (Engle et al. 1999). Interestingly, if these mice are made germ-free, they no longer develop colorectal cancer; this indicates that microbe-induced inflammation is crucial to the development of colon cancer (Engle et al. 2002). In the 129/Sv background, all Smad3^{null} mice develop tumors by 6 months of age, whereas in the hybrid 129/Sv;C57BL/6 background, only 30 % of these mice develop tumors (Zhu et al. 1998). Once again, the crucial role of the microenvironment is illustrated.
5. The last approach to developing digestive tract cancer predisposition models involves treating transgenic mice with external agents facilitating colorectal transformation. Indeed, although embedded in the body, the lumen of the digestive tract is in fact in direct contact with the external environment, rendering it more vulnerable to external aggressions. Fatty-acid-binding (FABP)-promoter-mediated Cre recombination makes it possible to target floxed alleles to the colonic crypts (Saam and Gordon 1999). FABP-Cre/TβRII-KO$^{L/L}$ mice displaying targeted inactivation of TβRII in the distal intestine do not develop tumors of the digestive tract but are more sensitive than their "control" littermates to colorectal carcinogenesis induced by azoxymethane (a chemical carcinogen) (Biswas et al. 2004). In a helicobacter-free environment, furthermore, Smad3^{null} mice do not develop colon cancer by the age of 9 months, and infection of these mice induces colon cancer in >50 % of the animals (Maggio-Price et al. 2006). Finally, it has been shown that TGF-β-KO^{het} mice developed more liver and lung tumors after treatment with diethylnitrosamine (DEN) (Tang et al. 1998), whereas transgenic mice overexpressing Smad3 are partially protected against DEN-induced liver carcinogenesis as a result of increased apoptosis (Yang et al. 2006). This again illustrates the crucial role of TGF-β-induced apoptosis in tumor suppression.

6.2.3 The Exocrine Pancreas

The pancreas comprises separate functional units that regulate two major physiological processes: digestion and glucose metabolism [for review, see (Gittes 2009)]. The exocrine pancreas consists of acinar and duct cells. The acinar cells produce digestive enzymes and constitute the bulk of the pancreatic tissue. They are organized into grape-like clusters at the smallest termini of the branching duct system. The ducts, which add mucous and bicarbonates to the enzyme mixture, form a network of increasing size, culminating in main and accessory pancreatic ducts that discharge their content into the duodenum. The endocrine pancreas, consisting of specialized cell types organized into compact islets embedded within the acinar tissue, secretes hormones (glucagon, insulin, pancreatic polypeptide, and somatostatin) into the bloodstream.

Pancreatic ductal adenocarcinoma (PDAC) is a very aggressive neoplasm, always lethal [for review, see (Kern et al. 2011)]. It is the fifth most common cause of death from cancer in the western world. At the time of diagnosis, the median age of PDAC-bearing patients is 65–70 years. PDAC affects the exocrine pancreas and accounts for ~80 % of pancreatic cancers. It arises from precursor lesions with differentiated ductal features. These precursor lesions are divided into three groups: pancreatic intraepithelial neoplasms (PanIN), intraductal papillary mucinous neoplasms (IPMN), and mucinous cystic neoplasms (MCN). Certain genetic alterations are recurrently found in sporadic and familial human PDAC, such as KRAS-activating mutations in over 90 % of cases and Ink4A/Arf- and TP53-inactivating mutations, each in about 50 % of cases [for review, see (Hansel et al. 2003) and (Landi 2009)]. The *SMAD4* gene is located at chromosomal locus 18q21, which is frequently lost in pancreatic cancer, especially in higher-grade lesions (Fukushige et al. 1998; Hahn et al. 1996; Schleger et al. 2000). Introduction of these mutations into mice has made it possible to create more than 20 models, recapitulating the whole spectrum of lesions observed in humans (according to the standard classification described in (Hruban et al. 2001), (Hruban et al. 2006) and http://pathology.jhu.edu/pancreas_panin/). Most of the models developed so far rely on targeted conditional expression in the Lox-Stop-Lox $KRAS^{G12D}$ (LSL-$KRAS^{G12D}$) knock-in mouse strain (Jackson et al. 2001), which bears a Cre-inducible allele targeting the endogenous KRAS locus with the most common alteration found in pancreatic cancers. Pancreas-specific activation of the LSL-$KRAS^{G12D}$ allele with the Pdx1-Cre (Gu et al. 2002) or Ptf1a/p48-Cre (Kawaguchi et al. 2002) allele induces the development of age-dependent precursor lesions (PanINs lesions in 100 % of animals) and PDAC after 1 year (in about 10 % of mice) (Hingorani et al. 2003). Combining the LSL-$KRAS^{G12D}$ allele with inactivation of the tumor suppressor Ink4A/Arf leads to very aggressive PDAC at a few weeks of age (median survival: approximately 2 months). This is the most robust and aggressive mouse model of PDAC developed to date (Aguirre 2003). This work and the unique opportunity offered by the Cre-lox system have prompted researchers to combine the $KRAS^{G12D}$ allele with many tumor suppressors inactivation and with oncogenic activations observed in humans (Bartholin 2012).

TGF-β signaling mutations combined with KRAS-activating mutations have emerged as very potent inducers of pancreatic cancer in mouse models, illustrating the tumor-suppressive role of TGF-β in this organ. These models provide a genuine opportunity to explore the complex role of TGF-β during carcinogenesis. Since Smad4 deletion is one of the most common genetic alterations in human PDAC, several mouse models have been developed where TGF-β signaling is specifically inactivated in the pancreas. Combined with KRAS activation, homozygous deletion of Smad4 induces cystic lesions, either IPMNs or MCNs (Bardeesy et al. 2006; Izeradjene et al. 2007; Kojima et al. 2007). Smad7 is a repressor of TGF-β signaling. Transgenic mice expressing Smad7 in the pancreas also develop PanINs (Kuang et al. 2006). Combined with TβRII inactivation targeted to the pancreas using the Ptf1a-Cre allele, $KRAS^{G12D}$ induces PDAC with a higher penetrance than observed with Smad4 (Ijichi et al. 2006), indicating that the tumor-suppressive function of TGF-β probably involves Smad4-independent mechanisms. Interestingly, PDAC cells in Ptf1a-Cre/LSL-$Kras^{G12D}$/$T\beta RII^{L/L}$ secrete high levels of several CXC chemokines, which in turn induce in the pancreatic stromal fibroblasts the expression of connective tissue growth factor (CTGF) , a profibrotic and tumor-promoting factor (Ijichi et al. 2011). This observation represents a novel evidence of the complex dialogue existing between the epithelial and the mesenchymal compartment during tumor progression.

Enlarged cells with vacuoles containing other cells have been observed by pathologists in human tumors for over 100 years. These “cells-in-cells” have notably been described in pancreatic cancer (Silverman et al. 1988; Tracey et al. 1984). At first, this cell cannibalism was hypothesized to be a pro-tumor mechanism, killing immune cells or feeding cancer cells upon starvation. More recently, a tumor-suppressive role for “cell-in-cells” process was proposed after the observation that cultured breast cancer cells could invade each other in vitro leading to a cell-death process that was called entosis or homotypic cell cannibalism (HoCC) (Overholtzer et al. 2007). Decreased HoCC has recently been reported in patients with metastasized PDAC (Cano et al. 2012), suggesting that HoCC plays a tumor-suppressive role in human pancreatic cancer. The HoCC decrease in the most aggressive PDACs is due to Nupr1. Nupr1is a stress protein activated at the transcriptional level by TGF-β (Pommier et al. 2012), overexpressed in late stages of PDAC and its metastases (Ito et al. 2005; Su et al. 2001a; b), involved in resistance to gemcitabine (the reference chemotherapy against PDAC) (Giroux 2006), and associated with a poor prognosis in PDAC patients (Hamidi et al. 2012). Nupr1 inhibits HoCC and enhances aggressiveness in PDAC by activating the EMT in response to TGF-β (Cano et al. 2012). Inactivation of Nupr1 in the $KRAS^{G12D}$/$Ink4A/Arf^{null}$ model (Pdx1-Cre/LSL-$KRAS^{G12D}$/Ink4A/Arf-$KO^{L/L}$/$Nupr1^{null}$ mice) results in a less aggressive phenotype characterized by enhancement of cell cannibalism. This validates the view that Nupr1 antagonizes cell cannibalism in vivo within the primary tumor. In Nupr1-depleted cells, unexpectedly, TGF-β stimulates HoCC (Cano et al. 2012). This suggests that HoCC is a tumor-suppressive program activated by TGF-β. The following model can be proposed: in the presence of Nupr1, TGF-β induces EMT and behaves as a tumor promoter; in the absence of Nupr1, TGF-β induces HoCC and behaves as a tumor suppressor.

Transcriptional intermediary factor 1γ (TIF1γ; alias, TRIM33/RFG7/PTC7/ectodermin) belongs to an evolutionarily conserved family of nuclear factors that have been implicated in stem cell pluripotency, embryonic development and tumor suppression (Hatakeyama 2011). Chromosomal breakpoints chromosome on 1p13.1 containing *TIF1γ* gene have been reported in acute megakaryocytic leukemias (Ng et al. 1999), osteochondromas (Sawyer et al. 2002), bronchial large cell carcinomas (Johansson et al. 1994), and childhood papillary thyroid carcinomas (Sawyer et al. 2002). Moreover, abrogation of the closely related *TIF1α* gene in mice caused hepatocellular carcinoma (Khetchoumian et al. 2007). These observations reinforce the idea according to which TIF1γ loss of function could play an active protective role during tumorigenesis. TIF1γ contributes to TGF-β signaling, although its precise functional role is not clear. Some data point toward TIF1γ as a negative regulator of the pathway through its capacity to mono-ubiquinate Smad4 and limit Smad4 nuclear accumulation (Dupont et al. 2005; 2009; Levy et al. 2007). In contrast, other studies have suggested that TIF1γ played an important positive role in transducing TGF-β signaling through its interaction with Smad2 and Smad3 (Bai et al. 2010; Doisne et al. 2009; He et al. 2006). For the reasons above mentioned, e.g., the involvement of TIF1γ in TGF-β signaling through its interaction with the Smad proteins, the location of TIF1γ at a locus that is altered in human tumors and the development of liver tumors in TIF1α knockout mice, a possible role of TIF1γ in pancreatic cancer was assessed. First, TIF1γ expression was reported to be markedly down-regulated in human pancreatic tumors (Vincent et al. 2009). Next, Pdx1-driven TIF1γ inactivation was shown to cooperate with the $KRAS^{G12D}$ oncogene in the mouse pancreas to induce exocrine pancreatic tumors (Vincent et al. 2009; Vincent et al. 2012). The relationship between *Tif1γ* and *Smad4* genes in pancreatic tumors was explored in double-null homozygous mutant mice in $KRAS^{G12D}$ background (Vincent et al. 2012) and revealed a pronounced genetic interaction between Smad4 and TIF1γ inactivation, the Pdx1-Cre; $KRAS^{G12D}$; Smad4-$KO^{L/L}$; TIF1γ-$KO^{L/L}$ (KSSTT) mice exhibiting accelerated tumor initiation (Vincent et al. 2012). The mechanism by which TIF1γ exerts its tumor-suppressive effect remains elusive. The increased tumor initiation observed when TIF1γ and Smad4 are simultaneously inactivated (compared to single gene inactivation) indicates that both genes may independently function as tumor suppressors of KRAS-driven pancreatic tumor. However, whether TIF1γ constrains transformation by modulating TGF-β functions, through mechanisms that are independent of Smad4, remains an open question and constitute a conceivable hypothesis consistent with the phenotype observed in Pdx1-Cre; LSL-$KRAS^{G12D}$; TRβII-$KO^{L/L}$ mice (Ijichi et al. 2006). Indeed, these mice, in which canonical and non-canonical TGF-β pathways are abrogated, present a phenotype resembling the one observed in KSSTT mice. Finally, it cannot be excluded that TIF1γ prevents transformation through mechanisms totally unrelated to TGF-β signaling, resulting from the modulation of general programs affecting broader genomic functions. For instance, TIF1γ and related family members were involved in chromatin remodeling, transcription elongation, and DNA repair (Agricola et al. 2011; Kepkay et al. 2011; Tsai et al. 2010). As briefly evocated in this chapter, after the malignant

transformation has occurred by bypassing its anti-proliferative and pro-apoptotic effects, TGF-β acquires oncogenic properties and eventually facilitates tumor progression. In some cases, TGF-β-mediated Smad4 activation may facilitate local invasion and metastatic dissemination by inducing EMT. In malignant tumors that retained a functional Smad4, it is possible that TIF1γ may behave as a tumor suppressor, through its capacity to mono-ubiquitinate Smad4 and limit its nuclear accumulation, thus limiting the aggressiveness of Smad4-positive cancer cells by compromising their capacity to undergo EMT. This hypothesis is supported both by in vitro and in vivo evidence. Indeed, it has been shown that TIF1γ could inhibit TGF-β-induced EMT of human mammary epithelial cells in culture (Hesling et al. 2011). In vivo, even if the double-null mice for TIF1γ and Smad4 develop pancreatic tumors with a shorter latency and a higher penetrance, these tumors present a well-differentiated phenotype contrasting with the poorly differentiated phenotype observed in TIF1γ null mice (Vincent et al. 2012). This phenotype may reflect the capacity of TIF1γ to inhibit Smad4-mediated EMT induced by TGF-β in later stages of tumorigenesis. TIF1γ inactivation cooperates with activated KRAS to promote the onset of poorly differentiated tumors in a Smad4-independent way. However, in vitro and in vivo evidence also supports that the absence of Smad4 could redirect TIF1γ-negative tumors toward a well-differentiated phenotype, consistent with a tumor-suppressive effect of TIF1γ relying on its capacity to inhibit Smad4-dependent EMT in late stage of tumorigenesis. Today, the exact mechanism by which TIF1γ behaves as a tumor suppressor independently of Smad4 in the early phase of transformation is still unclear, and an active tumor- suppressive role of TIF1γ in late stages of tumorigenesis that would constrain Smad4-induced EMT still needs to be demonstrated. The following model can be proposed to illustrate the complex role of TIF1γ in cancer (Fig. 6.2).

6.3 Conclusion

Genetically engineered mouse models bearing mutations found in human tumors have largely contributed to demonstrating in vivo the tumor-suppressive role of TGF-β. They have also provided crucial mechanistic information by revealing the crucial role of TGF-β-induced programs such as apoptosis and senescence in preventing transformation. These models have provided precious information on the capacity of TGF-β to limit progression of pre-neoplastic lesions towards aggressive tumors. By combining TGF-β signaling mutations with other mutations found in human tumors, it has been possible to generate mouse models that better mimic human diseases. These models have also enlightened the crucial role played by the microenvironment during carcinogenesis and have provided powerful systems to explore the origin of cancer cells. Finally, one can envision that these models will represent valuable tools for preclinical studies, an obligate step to develop new anticancer treatments.

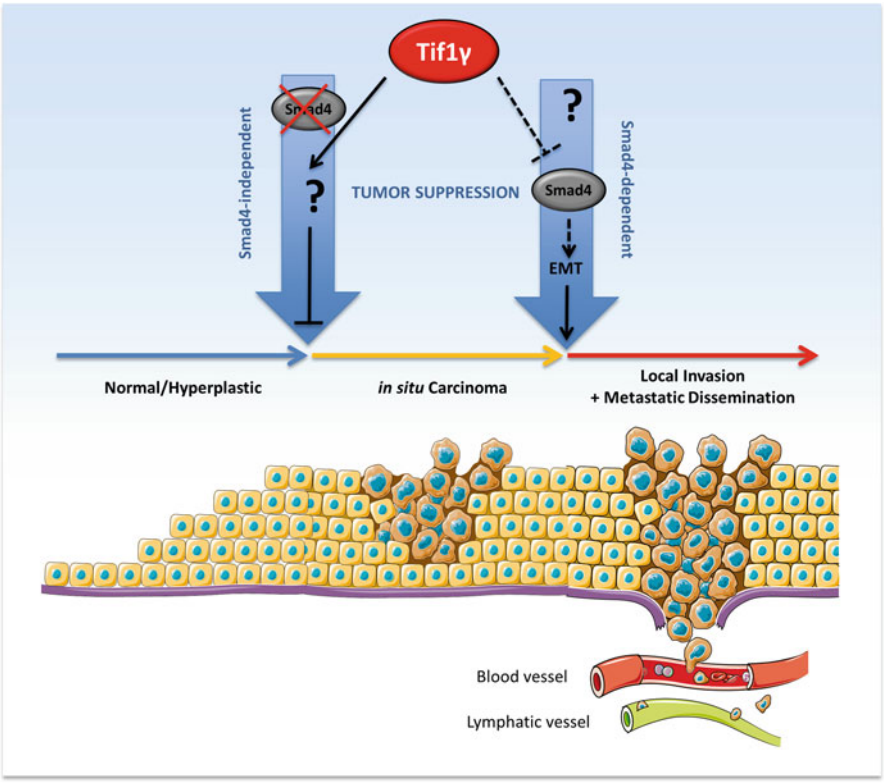

Fig. 6.2 Tumor-suppressive effects of TIF1γ. *Note*: The figures were illustrated using Servier Medical Art

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Chapter 7
The Multifunctional Roles of TGF-β in Navigating the Metastatic Cascade

Michael K. Wendt and William P. Schiemann

Abstract The role of TGF-β during tumorigenesis is best characterized by the diverse functions this multifunctional cytokine exhibits in early-stage versus late-stage cancers. For instance, during the initial stages of tumorigenesis, TGF-β uniformly acts as a potent tumor suppressor, even in low-grade carcinomas capable of evading the cytostatic activities of TGF-β. However, as carcinoma cells continue to evolve and progress towards more aggressive disease states they typically acquire the ability to surmount the last vestiges of the tumor suppressing activities of TGF-β, ultimately gaining a selective advantage that enables TGF-β to promote their metastatic progression and production of recurrent secondary tumor lesions that are refractory to standard chemotherapies. The molecular, cellular, and microenvironmental mechanisms that permit metastatic carcinoma cells to usurp and commandeer TGF-β for oncogenic activities are highly diverse and remain incompletely understood. Here we review recent advances that provide new insights into how aggressive carcinoma cell populations are selected to respond to the oncogenic activities of TGF-β, focusing specifically on its essential functions coupled to metastatic outgrowth and the acquisition of chemoresistance.

Keywords Chemoresistance • Epithelial-mesenchymal transition • Metastasis • Signal transduction • TGF-β

Abbreviations

ECM	Extracellular matrix
EGF	Epidermal growth factor

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EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
JNK	c-Jun N-terminal kinase
LAP	Latency-associated peptide
MAP kinase	Mitogen-activated protein kinase
MEC	Mammary epithelial cell
MET	Mesenchymal–epithelial transition
NF-κB	Nuclear factor-κB
PI3K	Phosphoinositide-3-kinase
TGF-β	Transforming growth factor-β
TβR-I	TGF-β type I receptor
TβR-II	TGF-β type II receptor
TβR-III	TGF-β type III receptors

7.1 Introduction

The major causes of cancer mortality are the processes of tumor recurrence and secondary tumor metastasis. Indeed, the 5-year survival rates for patients diagnosed with localized versus disseminated disease drops from (1) 99 to 23 % for breast cancers, (2) 90 to 12 % for colorectal cancers, (3) 98 to 15 % for melanomas, (4) 92 to 27 % for ovarian cancers, and (5) 100 to 29 % for prostate cancers (Siegel et al. 2012). Unfortunately, the multistep complexity of metastasis and its associated cascade of gene expression and repression has stymied our understanding of this inefficient and deadly process. Interestingly, recent findings have clearly established the TGF-β pathway as being an important mediator of carcinoma recurrence and metastasis (Taylor et al. 2010), which contrasts dramatically from its ability to suppress cellular transformation by preventing normal cells from proliferating uncontrollably, as well as by inducing their differentiation and apoptosis (Tian et al. 2010). Numerous studies over the last three decades have investigated the mechanisms that underlie these dichotomous functions of TGF-β in normal and malignant cells, a phenomenon that is collectively known as the "TGF-β Paradox." Although a single unifying sequelae operant in eliciting the "TGF-β Paradox" has yet to be defined, a significant body of work has demonstrated that aberrations within the TGF-β signaling system figure prominently in its acquisition of oncogenic activity in developing carcinomas (Rahimi and Leof 2007; Tian and Schiemann 2009; Wendt et al. 2012b). The propagation of TGF-β messages across plasma membranes is initiated by its binding to the TGF-β type II receptor (TβR-II), a Ser/Thr protein kinase that recruits, transphosphorylates, and activates the Ser/Thr protein kinase activity of the TGF-β type I (TβR-I) (Parvani et al. 2011; Tian et al. 2010). Once activated, TβR-I rapidly phosphorylates and activates the latent transcription factors, Smad2 and Smad3, which subsequently accumulate in the nucleus as part of heterocomplexes that contain Smad4 and govern gene expression in a cell- and context-specific manner (Feng

and Derynck 2005; Heldin and Moustakas 2012; Massague and Gomis 2006). The Smad2/3 pathway is commonly referred to as "canonical" TGF-β signaling, which is complemented by the ability of TGF-β to activate an ever expanding list of "non-canonical" signaling pathways, including (1) integrins and focal adhesion proteins; (2) PI3K and its effectors, AKT and mTOR; (3) NF-κB and inflammatory mediators; and (4) MAP kinases and their downstream effectors (Parvani et al. 2011; Taylor et al. 2010; Wendt et al. 2009a). Interestingly, a number of studies have demonstrated that genetic or epigenetic disruptions that elicit imbalances between these two core branches of TGF-β signaling represent the crux of the "TGF-β Paradox" and its ability to confer oncogenic functions to TGF-β in developing and progressing carcinomas (Tian and Schiemann 2009; Wendt et al. 2009b). Readers desiring additional information related to specific effector molecules and pathways of the TGF-β signaling system are directed to Chap. 1 herein, as well as to several recent reviews (Parvani et al. 2011; Taylor et al. 2010; Wendt et al. 2009a). Here we review recent findings that depict the function of TGF-β and its effector molecules as potent promoters of carcinoma progression and metastasis.

7.2 Epithelial–Mesenchymal Transition

One cannot address the role of TGF-β in mediating metastasis without first exploring the topic of epithelial–mesenchymal transition (EMT), which at its very core reflects the transdifferentiation of polarized epithelial cells into genetically and phenotypically distinct fibroblastoid-like cells (Kalluri and Weinberg 2009; Taylor et al. 2010; Wendt et al. 2009a). The epithelium is comprised of a monolayer of tightly packed epithelial sheets that not only form the skin but also line the internal cavities (e.g., ductal structures of the mammary gland, lung airways, and gastrointestinal tract), thereby providing a protective barrier to ward off environmental insults. Fully differentiated and polarized epithelial cells also exhibit a variety of specialized secretory, sensory, and glandular functions, many of which are regulated by TGF-β. In stark contrast, the mesenchyme functions to provide structural support for the epithelium, doing so via the production and secretion of the extracellular matrix (ECM), particularly collagens and fibronectins (Hay 2005). Moreover, although epithelial cells are highly confined and immobile, their mesenchymal counterparts are exceedingly mobile and invasive and thus are capable of exiting their associated epithelium and traversing through the underlying basement membrane and ECM (Micalizzi et al. 2010). As a single entity, the process of EMT is insufficient to drive carcinoma metastasis. In fact, EMT programs are a normal physiological process that has been hijacked by carcinoma cells to enable their dissemination to distant locales. In clarifying this issue, Kalluri and Weinberg recently subcategorized EMT into three distinct programs, including (1) Type 1 EMT, which occurs embryonically and developmentally during the formation of the endocardial cushion, neural crest formation, and palate closure and fusion; (2) Type 2 EMT, which occurs during tissue remodeling and repair, as well as during

inflammation-driven fibrotic reactions; and (3) Type 3 EMT, which occurs during the metastatic progression of carcinomas (Kalluri and Weinberg 2009). TGF-β is clearly a master regulator of all EMT subtypes and readers desiring a more thorough summary of the role of TGF-β in regulating EMT programs are directed to several comprehensive reviews (Heldin et al. 2012; Taylor et al. 2010; Wendt et al. 2009a). In the succeeding sections, we highlight the mechanisms whereby TGF-β induces EMT reactions, as well as discuss the utility of these events to identify and diagnose metastatic carcinomas.

7.2.1 Mechanisms of EMT Induced by TGF-β

Metastatic carcinoma cells that have emerged from EMT programs induced by TGF-β are endowed with several important characteristics, including heightened invasiveness, resistance to apoptosis, and the appearance of stem-like behaviors (Taylor et al. 2010; Wendt et al. 2009a; Yang and Weinberg 2008). The ability of TGF-β to induce EMT programs requires signaling inputs from both the canonical and noncanonical branches of the TGF-β signaling system, which converge in the nucleus to regulate the expression and activity of a variety of master transcriptional regulators of EMT (Taube et al. 2010). Included in this essential group of EMT transcription factors are members of the Snail (SNAI1 and SNAI2/Slug), ZEB (ZEB1 and ZEB2/SIP1), basic helix-loop-helix (Twist1 and Twist2), Six family of homeobox (Six1), and Forkhead (FOXC2), as well as members of the High Mobility Group proteins (HMGA2), which modify DNA structure to enhance transcription factor binding (Wendt et al. 2012b) (Fig. 7.1, *Mechanisms*). Interestingly, these transcription factors play essential roles during the performance of developmental Type 1 EMT programs, as well as during the initiation of oncogenic Type 3 EMT programs, suggesting that the inappropriate reactivation of embryonic EMT programs underlies the acquisition of metastatic phenotypes in response to TGF-β-driven EMT (Micalizzi et al. 2010). For instance, the basic helix-loop-helix transcription factor Twist1 plays a critical role in regulating developmental cell fate determination and differentiation reactions (Leptin 1991). However, consistent with the notion that metastatic carcinoma cells hijack physiological EMT programs during their systemic dissemination (Kang and Massague 2004), Twist1 expression is dramatically upregulated in metastatic breast cancer cells, a reaction that is regulated by TGF-β and essential for their metastasis in mice (Yang et al. 2004). Moreover, heterologous expression of Twist1 also empowers mammary epithelial cells with stem-like characteristics and increases their tumor-initiating capacity (Mani et al. 2008), thereby introducing the concept that EMT plays a number of diverse roles during carcinoma metastasis ranging from their increased invasiveness to their enhanced survival to their heightened chemoresistance (Fig. 7.1, *Consequences*). We recently added to this growing list of EMT functions by demonstrating that recombinant expression of Twist1 was sufficient to initiate the

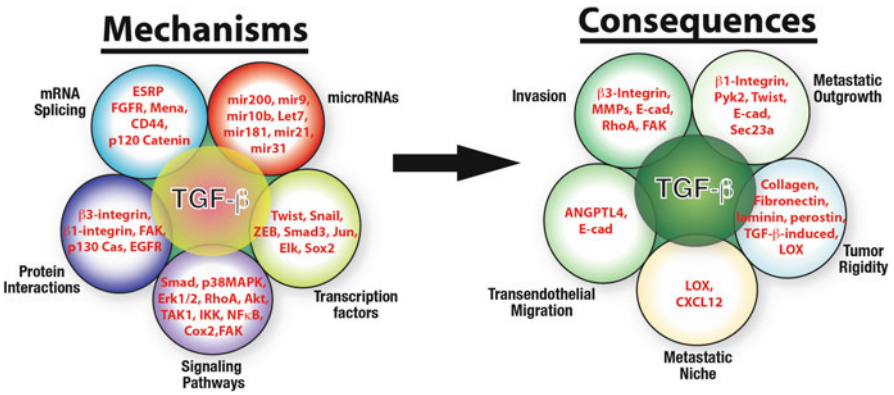

Fig. 7.1 The mechanisms and consequences of oncogenic TGF-β signaling during tumor progression and metastasis. TGF-β is a multifunctional cytokine that suppresses tumor formation by inhibiting cell proliferation and by stimulating cell differentiation or apoptosis. During carcinoma development and progression, TGF-β function is readily converted to that of a tumor promoter, leading to enhanced carcinoma cell EMT, invasion, and metastasis. Several of the established mechanisms of oncogenic TGF-β signaling are shown (*left*). The consequences of these events in relation to specific steps of the metastatic cascade are shown (*right*). At present, the precise "cause and effect" link between specific mechanisms and their associated consequences is expanding at a rapid rate; however, understanding how the consequences and pathophysiological readouts of oncogenic TGF-β signaling relate to multiple mechanistic events remains a daunting challenge to basic and translational researchers. See text for details

pulmonary outgrowth of disseminated breast cancer cells that otherwise succumb to systemic dormancy (Wendt et al. 2011b) (Fig. 7.1, *Consequences*). Along these lines, recent findings indicate that transcription factors coupled to the induction of EMT programs work in concert both directly and indirectly to bring about EMT reactions. For instance, Casas and colleagues (Casas et al. 2011) recently demonstrated that the ability of Twist1 to repress E-cadherin expression during the EMT and metastasis of breast cancer cells was absolutely dependent upon its stimulation of Snail2 (Slug) (Fig. 7.1, *Mechanisms*). Similar to the response of Twist1 to TGF-β, members of the Snail family of zinc finger transcription factors (SNAI1-3) are also induced in cells stimulated by TGF-β (Horiguchi et al. 2008), leading to the direct transcriptional repression of a variety of epithelial genes, including E-cadherin (Hajra et al. 2002) (Fig. 7.1, *Consequences*). Finally, TGF-β readily stimulates the expression of high mobility group A2 (HMGA2), which promotes EMT programs by upregulating the expression of Snail1, Snail2, and Twist, which collectively converge in downregulating that of E-cadherin (Thuault et al. 2006) (Fig. 7.1). Future studies need to define spatiotemporal regulation of these EMT transcription factors by TGF-β in developing and progressing carcinomas and, more importantly, establish the specific steps in the metastatic cascade that are dependent upon these transcription factors.

7.2.2 *Defining the Metastatic EMT Morphology*

To date, EMT programs have been predominantly studied in the context of traditional 2D-culture systems, whose artificial nature imposes significant cellular and genetic adaptations necessary for cells to thrive in this biomechanically rigid microenvironment. Indeed, the prominent feature of transitioning epithelial cells in 2D-cultures is their formation of actin-rich filopodia and actin stress fibers as they acquire fibroblastoid-like morphologies. Teleologically, these morphological features are in keeping with the acquisition of motile, invasive, and metastatic phenotypes by carcinoma cells. Unfortunately, these actin-based architectures are an artifact of propagating mesenchymal cells on rigid 2D-cultures and, in fact, are not observed in mesenchymal cells in vivo (Hay 2005). Similarly, the advent of 3D-organotypic culture systems and the propagation of post-EMT cells in these systems have also called into question the precise morphology exhibited by fully transitioned carcinoma cells in vivo, particularly in tumor microenvironments that possess elevated expression and activity of TGF-βs. Indeed, we recently established that applying breast cancer cells to 3D-organotypic cultures results in the formation of intricate branched structures that readily assume dense multicellular spherical structures in response to TGF-β (Wendt et al. 2010, 2011a, b, 2012a). Importantly, the formation of these "invasospheres" transpires through an EMT-dependent mechanism and reflects the ability of TGF-β to promote the invasion and metastasis of these same breast cancer cells in mice (Wendt et al. 2010, 2012a). Collectively, these findings suggest that the histological association of sarcomatoid morphologies with EMT phenotypes may be misleading and misguiding when attempting to identify post-EMT carcinoma cells in vivo. In an effort to alleviate the current controversies surrounding EMT phenotypes in vivo, future studies clearly need to identify the morphologies and features of post-EMT cells at specific steps of the metastatic cascade, as well as to determine their ability to predict the overall survival and clinical outcome of patients with metastatic disease.

7.2.3 *The Diagnostic Value of EMT*

The crosstalk and linkages between the aforementioned EMT transcription factors have greatly increased our understanding of how TGF-β regulates epithelial plasticity in normal and malignant cells. However, while the list of "master regulators" of EMT programs continues to expand at an alarming rate (Fig. 7.1; *Mechanisms*), the utility of these transcription factors to function as diagnostic or predictive biomarkers of metastatic progression, as well as novel therapeutic targets to alleviate carcinoma dissemination is compromised at an equally alarming rate. Indeed, single cell analysis of circulating tumor cells has shown that these cells simultaneously express epithelial and mesenchymal markers, suggesting the existence of metastable and dynamic EMT phenotypes in carcinoma cells that have escaped the primary tumor

(Armstrong et al. 2011). Given the difficulty to pathologically identify EMT in vivo and the plasticity of EMT programs in highly malignant cells, the overall clinical utility and effectiveness of detecting and targeting of post-EMT carcinoma cells harboring mesenchymal characteristics remains to be determined. For instance, EMT and its counterpart, mesenchymal–epithelial transition (MET), are transient processes that likely take place numerous times during the evolution of a tumor and its dissemination to vital organs. Indeed, several recent studies that examined the plasticity of a variety of breast and other carcinoma cell progression series indicate that the maintenance of a perpetual mesenchymal-state limits the overall tumorigenicity and aggressiveness of fully transitioned cells in response to TGF-β (Korpal et al. 2011; Wendt et al. 2011a, b). As such, evolving the capacity to undergo successive cycles of EMT:MET selects for carcinoma cells that possess the highest metastatic potential. Moving forward, future studies need to identify and validate novel biomarkers capable of differentiating primary, secondary, and tertiary EMT states, which may provide novel diagnostic and therapeutic regimens to alleviate metastatic disease.

7.3 Emerging Paradigms in TGF-β-Mediated Gene Regulation

7.3.1 Diversity in Smad Signaling

The specific set of genes targeted by canonical TGF-β signaling varies greatly in a cell- and context-specific manner. For example, the expression of a particular gene may be induced by TGF-β in fibroblasts, whose proliferation is stimulated by TGF-β; however, this same gene may be potently downregulated by TGF-β in epithelial cells, whose proliferation is inhibited by TGF-β. This diversity in TGF-β signaling has historically been believed to reflect the differential ability of TGF-β to couple to its canonical and noncanonical effectors in distinct cells types, as well as the differential influence of heterologous signals that impinge upon TGF-β effector molecules. However, recent findings have demonstrated an important alternative mechanism, namely the differential and physical interactions between Smad3 with specific "master" transcription factors, including Oct4 in embryonic stem cells (ESCs), Myod1 in myotubes, and PU.1 in pro-B cells (Mullen et al. 2011), thereby enabling Smad3 and TGF-β to direct cell-fate outcomes during tissue morphogenesis and remodeling (Fig. 7.1, *Mechanisms*). Canonical TGF-β signaling is also impacted by the direct phosphorylation of Smad2/3 by a variety of Ser/Thr protein kinases, including members of the CDK and MAP kinase families, GSK-3β, PKC, CK1γ2, and several others (Hayashida et al. 2003; Matsuura et al. 2005; Matsuzaki 2011; Wrighton et al. 2009) (Fig. 7.1, *Mechanisms*). Secondary phosphorylation of Smad2/3 by these protein kinases can alter the subcellular localization, stability, and transcriptional activity of these canonical TGF-β effectors, particularly their

regulation of the expression of metastasis-associated genes (Hayashida et al. 2003). Along these lines, the amplified activation of oncogenic signaling systems (e.g., receptor tyrosine kinases, Ras, PI3K, etc.), which modulate the activation status of Smad2/3-directed protein kinases, further dysregulates the repertoire of canonical TGF-β signaling and enables developing carcinoma cells to evade the tumor suppressing activities of TGF-β (Fig. 7.1, *Mechanisms*).

7.3.2 Regulation of MicroRNAs

In addition to traditional modes of gene expression regulated by canonical and non-canonical TGF-β signaling systems, recent studies have established TGF-β as a potent regulator of microRNAs in developing carcinomas, particularly during their acquisition of metastatic phenotypes. The linkage of microRNAs to oncogenic TGF-β signaling was initially described by its regulation of the miR-200 family of microRNAs, which function in suppressing the expression of the EMT transcription factors, ZEB1 and ZEB2 (SIP1) (Gregory et al. 2008; Korpal et al. 2008) (Fig. 7.1, *Mechanisms*). During EMT programs and metastatic progression, TGF-β promotes the downregulation of miR-200 family members, which stabilizes expression of ZEB1 and ZEB2 and enables their binding to and repression of the *Cdh1* (E-cadherin) promoter, resulting in a loss of E-cadherin expression in transiting carcinoma cells (Fig. 7.1, *Consequences*). Intervening studies have since expanded the repertoire of gene transcripts that are targeted and regulated by the miR-200 family, which are remarkably correlated with those targeted and regulated by TGF-β (Howe et al. 2011; Schliekelman et al. 2011). Interestingly, a significant portion of genes targeted by members of the miR-200 family are coupled to the production of proteins operant in producing or sensing alterations in the tumor microenvironment. Included in this growing list of microenvironmental sensors are the ECM molecules fibronectins, collagens, and laminins, as well as cell surface receptors that connect the extracellular and intracellular milieu, such as integrins and growth factor receptors (Fig. 7.1, *Consequences*). Importantly, we recently demonstrated that aberrant expression of these factors underlies the initiation of the "TGF-β Paradox" and promotes the acquisition of oncogenic TGF-β signaling in developing and progressing carcinomas (Galliher and Schiemann 2006, 2007; Galliher-Beckley and Schiemann 2008; Taylor et al. 2011; Wendt and Schiemann 2009; Wendt et al. 2009b). Additionally, recent findings implicate a powerful feed-forward signaling loop between TGF-β and miR-200 family members in driving oncogenic EMT and carcinoma metastasis (Gregory et al. 2011). Collectively, these findings establish miR-200 family members as tumor suppressors capable of preventing oncogenic TGF-β signaling and its induction of EMT in normal epithelial cells (Schliekelman et al. 2011). Surprisingly, two recent studies demonstrated that highly metastatic 4T1 breast cancer cells are more epithelial-like as compared to their isogenic and non-metastatic 4T07 counterparts (Korpal et al. 2011; Wendt et al. 2011a, b). Amongst the differences between these two cell types is the reestablishment of miR-200 in

4T1 cells, an event that aids in the production and secretion of metastasis-promoting proteins necessary for the outgrowth of macroscopic metastases (Korpal et al. 2011) (Fig. 7.1, *Consequences*).

Besides regulating the expression of miR-200 family members, TGF-β also dictates the activities of microRNAs by promoting their processing. For instance, Smad2/3 interacts physically with DROSHA, which enhances the conversion of primary miR-21 transcripts into their pre-miR-21 counterparts in a Smad4-independent manner (Davis et al. 2008) (Fig. 7.1, *Mechanisms*). The coupling of TGF-β to EMT programs also proceeds via its ability to upregulate the expression of miRs 21 and 31, resulting in the downregulation of Tiam1 (Cottonham et al. 2010; Zavadil et al. 2007), and miR-155, leading to the repression of RhoA expression (Kong et al. 2008) (Fig. 7.1, *Consequences*). Finally, we have identified miR-181a as a TGF-β-inducible "metastamir" that enhances breast cancer metastasis by facilitating the downregulation of the pro-apoptotic molecule, Bim, thereby conferring miR-181a-expressing carcinoma cells resistance to anoikis (Taylor et al. 2013) (Fig. 7.1, *Consequences*).

7.3.3 Alternative Splicing

The splicing of mRNAs is a post-transcriptional modification that functions to dramatically increase the diversity of gene products produced from a static genome. In fact, transcription of the vast majority of the genes in the genome (~90 %) gives rise to two or more protein products due to the process of alternative splicing. Recently, TGF-β has been suggested to play a major role in mRNA splicing, particularly in developing carcinomas through its ability to suppress the expression of epithelial splicing regulatory proteins (ESRPs), which are a class of RNA-binding proteins that govern the splicing of epithelial gene transcripts (Horiguchi et al. 2012; Warzecha et al. 2010). Indeed, the ability of distinct protein variants to impact cancer development, EMT, and metastasis is rapidly coming to the forefront of cancer biology, including that regulated by TGF-β. For instance, the initiation of alternative gene splicing programs that transpire during EMT reactions driven by TGF-β results in part from its ability to downregulate the expression of ESRP2 via a ZEB1/δEF1- and ZEB2/SIP1-dependent manner (Horiguchi et al. 2012) (Fig. 7.1, *Mechanisms*). Perhaps the best studied example of differential gene splicing governed by TGF-β relates to its regulation of the four member fibroblast growth factor receptor (FGFR) family, of which FGFRs 1–3 are subject to alternative splicing that greatly enhances isoform diversity and function (Fig. 7.1, *Mechanisms*). TGF-β regulates two major splicing events in FGFRs. First, alternative splicing of the extracellular Ig-like domain I (α-loop) results in either the inclusion or the exclusion of the first Ig-like domain. Importantly, exclusion of Ig-like domain I drastically increases the affinity of the FGFR to bind members of the FGF ligand family, which currently comprises 22 individual cytokines (Jain and Turner 2012; Wesche et al. 2011). Second, alternative splicing of the distal portion of Ig-like domain III dictates the specificity of

FGFRs to bind specific FGF ligands (Holzmann et al. 2012; Jain and Turner 2012; Werner et al. 1992). Inclusion of exon 8 results in the production of FGFR-IIIb isoforms capable of binding to FGF7 (KGF) and FGF10, while inclusion of exon 9 results in the production of FGFR-IIIc isoforms that selectively bind FGF2 and FGF4 (Holzmann et al. 2012; Jain and Turner 2012; Werner et al. 1992). Interestingly, FGFR-IIIb and -IIIc isoforms are preferentially expressed in epithelial and mesenchymal cells, respectively, and the induction of EMT programs by TGF-β is sufficient to switch epithelial FGFR-IIIb expression to that of its -IIIc counterpart in post-EMT cells (Shirakihara et al. 2011) (Fig. 7.1, *Mechanisms*). It should be noted, however, that the metastatic cells that exhibit epithelial morphologies and characteristics retain FGFR-IIIc expression (Chaffer et al. 2006), suggesting the presence of a metastable phenotype in metastatic cells that have emerged from successive rounds of EMT:MET. Future studies need to further address the consequences of FGFR splicing during TGF-β-driven EMT and metastasis, particularly during the latter stages of metastatic progression and emergence from micrometastatic dormancy (Korpal et al. 2011; Wendt et al. 2011a, b), and as a potential predictive biomarker and therapeutic target for recurrent metastatic tumors.

7.4 TGF-β and the Metastatic Cascade

7.4.1 Primary Tumor Local Invasion

The initial step of the metastatic cascade requires tumor cells to exit the confines of a primary tumor and invade into the surrounding tissue. TGF-β plays a critical role in stimulating tumor cell invasion through its ability to upregulate the expression and activity of extracellular proteases charged with degrading tumor ECM and supporting basement membrane. For instance, TGF-β robustly induces the expression of several matrix metalloproteinases (MMPs), including MMPs 2, 3, 7, 13, MT1, and especially MMP-9 (Wendt et al. 2009a) (Fig. 7.1, *Consequences*). Although the signaling mechanisms that couple TGF-β to MMP expression remain to be determined definitively, recent studies have implicated the noncanonical effector, TAK1 and its downstream targets as mediators of MMP-9 expression stimulated by TGF-β (Safina et al. 2008) (Fig. 7.1, *Mechanisms*). In general, genetic depletion or pharmacologic inactivation of MMPs drastically decreases the ability of TGF-β to stimulate the invasion of metastatic carcinoma cells (Wendt et al. 2009a). Besides their ability to degrade ECM during carcinoma invasion, MMPs also cleave LAP (latency-associated peptide) that permits the liberation of biologically active TGF-β from inactive ECM depots, thereby initiating a positive feed-forward loop coupled to elevated autocrine TGF-β signaling (Rifkin 2005; Taylor et al. 2010).

TGF-β also enhances carcinoma invasiveness by altering their production of and response to ECM molecules within the developing tumor microenvironment (Stover

et al. 2007). Indeed, when stimulated by TGF-β, metastatic carcinoma cells elevate their production of a wide array of ECM proteins, including collagens (Ignotz and Massague 1986), fibronectin (Ignotz and Massague 1986), periostin (Wen et al. 2010), and laminins (Giannelli et al. 2005; Kumar et al. 1995), as well as a newly described protein known as TGF-β-induced [TGFBI; (Nummela et al. 2012)] that limits cell adhesion to structural ECM proteins (Fig. 7.1, *Consequences*). Integrins are heterodimeric transmembrane receptors that function as conduits capable of linking the ECM to intracellular signaling and cytoskeletal systems (Hall 2009; Zutter 2007). Principle players involved in TGF-β-mediated response to ECM proteins are αvβ3 integrins, whose expression is robustly induced by TGF-β via a Smad2/3-independent mechanism (Galliher and Schiemann 2006; Pechkovsky et al. 2008). Amplified expression of αvβ3 integrin results in its indirect interaction with TβR-II, a reaction mediated by focal adhesion kinase (FAK); (Wendt and Schiemann 2009). Importantly, the incorporation of αvβ3 integrin within activated TGF-β receptor complexes enables Src to phosphorylate TβR-II at Tyr284 (Galliher and Schiemann 2007). This phosphorylation event facilitates the binding of Grb2 and ShcA to TβR-II leading to amplified noncanonical TGF-β signaling through MAP kinases (Galliher et al. 2006; Galliher and Schiemann 2007; Galliher-Beckley and Schiemann 2008). Additionally, αvβ3 integrin:TβR-II complexes couple TGF-β to FAK (Wendt and Schiemann 2009), p130Cas (Wendt et al. 2009b), and Pyk2 (Wendt et al. 2012a), which further enhance the coupling of TGF-β to several noncanonical effector molecules, including NF-κB (Neil and Schiemann 2008), Cox-2 (Neil et al. 2008), and PGE2 (Tian and Schiemann 2010). In fact, pharmacologic or genetic inactivation of FAK effectively blocks the invasion and metastasis of breast cancer cells (Wendt and Schiemann 2009), while similar approaches against the FAK target, p130Cas, only inhibit breast cancer invasion and primary tumor dissemination without affecting the overall extent of pulmonary metastasis (Wendt et al. 2009b). Thus, these findings point to the existence of novel rate-limiting steps during initiation of the metastatic cascade in response to TGF-β.

Finally, β3 integrin also governs oncogenic TGF-β signaling by interacting physically with EGFR, a reaction that enables fibronectin to transactivate EGFR in the absence of EGF ligands (Balanis et al. 2011). Along these lines, we showed that FAK serves as a bridge that links EGFR with TβR-II in post-EMT cells, leading to their acquisition of invasive phenotypes when stimulated with EGF (Wendt et al. 2010). Finally, β3 integrin binds FGF and the co-expression of both molecules is highly predictive for lung cancer recurrence (Massabeau et al. 2009; Mori et al. 2008). Collectively, these studies establish the unique ability of integrins, particularly β3 integrin, to influence both the mechanisms of TGF-β signaling and the metastatic consequences of these events (Fig. 7.1). Moreover, these findings highlight the ability of oncogenic TGF-β signaling to cooperate with and activate a cascade of aberrant growth factor signaling events that coalesce to promote several aspects of the metastatic cascade.

7.4.2 Establishment of the Metastatic Niche

The pathophysiology of TGF-β in developing and progressing carcinomas in many respects reflects changes in its expression and bioavailability within the tumor microenvironments, as well as changes within the biomechanics of the tumor microenvironment. For instance, we recently established that TGF-β upregulates the expression of lysyl oxidase (LOX) (Taylor et al. 2011), a secreted copper amine oxidase that enzymatically crosslinks collagen to elastin (Erler et al. 2009; Erler and Giaccia 2006). The outcome of these LOX-mediated crosslinking reactions produces hydrogen peroxide as a by-product that serves as a novel second messenger for TGF-β (Taylor et al. 2011), as well as generates the rigid, palpable characteristics of carcinomas as compared to their surrounding normal tissues (Butcher et al. 2009) (Fig. 7.1, *Consequences*). Collectively, these events drive the oncogenic activities of TGF-β, including its stimulation of EMT, invasive, and metastatic phenotypes (Taylor et al. 2010, 2011) in part by amplifying integrin-mediated signaling events (Levental et al. 2009) and establishing the premetastatic niche (Erler et al. 2009). The latter activity reflects the ability of crosslinked collagens to serve as homing and docking sites during the recruitment of myeloid progenitors to future metastatic sites. Importantly, the recruitment of myeloid progenitors and formation of a premetastatic niche at peripheral sites is controlled in part by TGF-β via its regulation of stromal cell derived factor-1 [SDF-1; (Yang et al. 2008)] (Fig. 7.1, *Consequences*). Taken together, these studies suggest a model wherein aberrant TGF-β signaling upregulates primary tumor expression of LOX, while simultaneously downregulating that of SDF-1, leading to the homing of myeloid progenitors and premetastatic niche formation at peripheral sites of high SDF-1 expression, such as the lungs, brain, and bone marrow.

7.4.3 Transendothelial Cell Migration

Upon exiting the primary tumor, carcinoma cells must intravasate and extravasate the blood or lymphatic vessels before they can invade into a secondary tumor site (Nguyen et al. 2009). The processes of intravasation and extravasation are reminiscent of those employed by immune cells, whose ability to perform these tasks remain incompletely understood. Not surprisingly, even less is known mechanistically with regard to how metastatic cells emulate these events. Recently, TGF-β was shown to induce angiopoietin-like 4 (ANGPTL4) expression, which enables breast cancer cells to disrupt the vasculature and transit through the pulmonary endothelium (Padua et al. 2008) (Fig. 7.1, *Consequences*). Along these lines, the downregulated expression of E-cadherin in post-EMT prostate cancer cells facilitates their migration through endothelial cell layers (Drake et al. 2009) (Fig. 7.1, *Consequences*). Taken together, these findings highlight two consequences of TGF-β signaling that are operant in facilitating transendothelial migration of carcinoma cells during their dissemination. Future studies need to bolster our understanding of transendothelial

cell migration and its regulation by TGF-β, as well as to determine the therapeutic potential of targeting the effectors operant in this process as a novel means to prevent carcinoma cell seeding at secondary tumor sites.

7.4.4 Secondary Tumor Outgrowth

Following their dissemination and invasion into secondary tumor sites, metastatic carcinoma cells must surmount the tendency to undergo systemic dormancy, which may represent the most significant obstacle of the metastatic cascade (Brackstone et al. 2007). Indeed, estimates indicate that even small tumors (~1 cm) can release more than one million cells each day into the blood stream (Chiang and Massague 2008; Talmadge and Fidler 2010), signifying that the metastatic cascade is a highly inefficient process that is subject to two major limitations, namely the inability of disseminated cells to (1) escape the lethality of vascular shear stress and (2) circumvent metastatic dormancy. With respect to the latter phenomenon, it is important to remember that metastatic cells typically exit biomechanically rigid primary tumors and subsequently take up residence in new organs that are noticeably more compliant (Butcher et al. 2009). Indeed, using 3D-organotypic cultures that recapitulate rigid primary tumor and compliant pulmonary microenvironments, we observed that a compliant microenvironment could restore some of the tumor suppressing activities of TGF-β to highly metastatic breast cancer cells (Allington et al. 2009; Taylor et al. 2011), indicating that the extent to which TGF-β exhibits oncogenic activities is directly proportional to the degree of microenvironmental rigidity. Interestingly, metastatic cells that have completed an EMT program readily circumvent the suppressive activities of compliant microenvironments and instead utilize TGF-β as a means to promote their outgrowth (Wendt et al. 2011a, b, 2012a). Mechanistically, increased tissue compliance inhibits the coupling of TGF-β to Smad4 (Korpal et al. 2009; Wendt et al. 2011a, 2012a) (Fig. 7.1, *Mechanisms*), which prevents the expression of Pyk2 necessary to mediate escape from metastatic dormancy (Wendt et al. 2012a) (Fig. 7.1, *Consequences*). These events are also regulated by the reciprocal expression patterns of E-cadherin and β1 integrin, such that dormant cells are typically E-cadherin high, β1 integrin low, while outgrowth proficient cells are typically β1 integrin high, E-cadherin low (Barkan et al. 2008, 2010; Shibue and Weinberg 2009; Wendt et al. 2011a, 2012a). Importantly, we determined that EMT programs represent the molecular switch between metastatic dormancy and proficiency due to the downregulation of E-cadherin expression, which interacts in a heterotypic manner with the extracellular domain of β1 integrin (Whittard et al. 2002), downregulating its expression and that of Pyk2, collectively preventing the initiation of proliferative programs (Wendt et al. 2011a, b, 2012a). Likewise, the expression and activity of FAK has also been implicated in the initiation of metastatic outgrowth (Shibue and Weinberg 2009), and as such, FAK and Pyk2 represent essential TGF-β effectors that link altered integrin expression and ECM biomechanics with the micrometastatic machinery operant in driving secondary tumor formation (Fig. 7.1, *Consequences*).

7.5 Conclusions

In years past, cancer biologists have analyzed tumor development and progression primarily using a "tumoricentric" approach—i.e., focusing on studies that determine the molecular and genetic basis of how tumors are initiated, progress, and ultimately impact their surroundings. New studies emphasizing translational and tissue-based research approaches have ushered in a new arena of cancer biology aimed at understanding how clinical regimens and targeted therapies impact the aforementioned processes of tumor development and metastatic progression. A primary focus of these analyses deals with the development of chemoresistance, which typically reflects three distinct subtypes of acquired resistance: (1) development of secondary mutations within the drug target; (2) compensatory activation and/or amplification of additional growth promoting pathways; and (3) acquisition of EMT phenotypes following drug treatment (Baum et al. 2008; Sharma et al. 2010; Singh and Settleman 2010). With respect to the third mechanism, recent studies suggest that chemoresistant cells do in fact display a mesenchymal phenotype indicative of EMT programs. Thus, the principal question moving forward relates to whether chemoresistant carcinoma cells are actually derived from a low-frequency resident population that was selected out of the bulk tumor, or whether these chemoresistant variants emerged from their performance of an EMT program? Although additional experimentation is clearly warranted, the chemoresistance of metastatic carcinoma cells has been associated with the acquisition of EMT programs (Creighton et al. 2009; Farmer et al. 2009; Sharma et al. 2010; Taube et al. 2010), particularly that driven by TGF-β (Mani et al. 2008; Shipitsin et al. 2007). Likewise, TGF-β has also been observed to drive an EMT program coupled to radioinsensitivity (Barcellos-Hoff and Akhurst 2009). Collectively, these findings suggest that co-administering TGF-β antagonists with targeted chemotherapies may provide novel inroads to reverse this consequence of EMT programs, and in doing so, may sensitize late-stage carcinoma cells not only to newly developed targeted chemotherapies but also to traditional genotoxic and cytotoxic agents. Future studies need to address this hypothesis and determine the most effective drug and dosing combinations needed to eradicate these recalcitrant tumor subpopulations. Likewise, future studies need to continue elucidating the molecular mechanisms whereby TGF-β drives carcinoma development and metastatic progression, paying particular attention at determining how the flux through the TGF-β signaling system changes at distinct steps of the metastatic cascade, and at defining novel TGF-β-based biomarkers capable of predicting clinical outcomes, directing treatment regimens, and serving as novel drug targets.

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Chapter 8
TGF-β Signaling in Leukemogenesis

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Abstract Transforming growth factor-β (TGF-β) signaling plays diverse roles in regulating cell proliferation, differentiation, and "stemness" in a cell context-dependent manner. In the first section of this chapter, we outline the genetic changes that attenuate TGF-β signaling in patients with acute myelogenous leukemia (AML), or the blast crisis phase of chronic myelogenous leukemia (CML), or pediatric acute lymphoblastic leukemia (ALL). In the second section, we discuss recent advances in stem cell research indicating that TGF-β signaling does not always suppress leukemogenesis. In fact, TGF-β signaling paradoxically sustains the survival and resistance to therapy of the CML stem cells that are responsible for disease recurrence in CML patients treated with tyrosine kinase inhibitors (TKIs). We examine evidence implicating TGF-β and its downstream effectors Akt and FOXO in the in vivo maintenance of the self-renewal ability of both TKI-resistant CML stem cells and the normal hematopoietic stem cells (HSCs) from which they are derived. Increased knowledge of the complex effects of TGF-β signaling may lead to improved diagnostic and therapeutic tools that can benefit leukemia patients.

Keywords TGF-β • Smad • FOXO • CML stem cells • TKI-resistance

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Abbreviations

ALL	Acute lymphoblastic leukemia
AML	Acute myelogenous leukemia
ANLL	Acute nonlymphoblastic leukemia
APL	Acute promyelocytic leukemia
ATRA	All-*trans* retinoic acid
BMP	Bone morphogenetic protein
Bmpr1a	BMP receptor type 1A
CCyR	Complete cytogenetic response
CML	Chronic myelogenous leukemia
cPML	Cytoplasmic isoform of PML
FAB	French–American–British
GFAP	Glial fibrillary acidic protein
HSC	Hematopoietic stem cell
KIR	Killer immunoglobulin-like receptor
L-GMP	Leukemic-granulocyte-macrophage progenitor
LRC	Lipid raft clustering
LSK	$Lineage^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$
MEF	Mouse embryonic fibroblast
MPN	Myeloproliferative neoplasm
NK	Natural killer
PI3K	Phosphatidylinositol-3-OH kinase
PML	Promyelocytic leukemia
RARα	Retinoic acid receptor α
ROS	Reactive oxygen species
SARA	Smad anchor for receptor activation
SCL	Stem cell leukemia
SOD	Superoxide dismutase
TGF-β	Transforming growth factor-β
TKI	Tyrosine kinase inhibitor
WHO	World Health Organization

8.1 Genetic Alterations Affecting the TGF-β-Smad Pathway in Leukemia Patients

The transforming growth factor-β (TGF-β) family of highly conserved and highly homologous secreted polypeptides (Massagué et al. 1990) was described in Chap. 1. Briefly, TGF-β molecules bind to two heteromeric cell surface receptors with serine/threonine kinase activity: TGF-β receptor type I (TGF-βRI) (also known as activin receptor-like kinase 5; ALK5) and TGF-β receptor type II (TGF-βRII) (Massagué 2000). Binding of TGF-β to TGF-βRII triggers the recruitment and

activation of TGF-βRI, which phosphorylates downstream Smad proteins (Heldin et al. 1997). That TGF-β signaling is involved in leukemogenesis has been known (Blank and Karlsson 2011; Dong and Blobe 2006; Kim and Letterio 2003; Larsson and Karlsson 2005). It has been known that TGF-β signaling pathway is altered in hematological malignancies such as acute myelogenous leukemia (AML) and final fatal phase of chronic myelogenous leukemia (CML) termed "blast crisis." AML is a heterogeneous clonal disease characterized by rapid accumulation of abnormal immature white blood cells in the bone marrow and peripheral blood. Although chronic phase CML is a relatively benign myeloproliferative neoplasm (MPN), CML patients in blast crisis reveal rapid proliferation of immature leukemic blasts which are resistant to therapy. In addition, genetic changes that attenuate TGF-β signaling also drive the excessive cell proliferation that characterizes acute lymphoblastic leukemia (ALL) originated from T-cell and B-cell lineage.

In this section of this chapter, we examine this assumption in the context of major chromosomal alterations that affect TGF-β signaling in human leukemia patients.

8.1.1 Acute Myelogenous Leukemia

8.1.1.1 t(8;21)(q22;q22)-Positive AML

The traditional French–American–British (FAB) classification system used to diagnose AML patients in twentieth century was based on cellular morphology and cytochemistry. Starting in 2001, a new World Health Organization (WHO) classification system that takes into account cell morphology, patient and clinical factors, and genetic and chromosomal alterations has been used to provide a scientific basis for AML diagnosis (Jaffe et al. 2001; Swerdlow et al. 2008). The chromosomal translocation t(8;21)(q21.3;q22.12), which generates the AML1–ETO fusion oncoprotein, is a causal genetic change in cases of "immature" type AML (defined as M0 in the FAB system). The normal AML1 protein, also known as Runx1, CBFA2 or PEBP2αB, is a member of the *runt* family of transcription factors. In mammalian embryos, AML1 is critical for the development of definitive hematopoiesis. In adults, AML1 is dispensable for the maintenance of normal hematopoietic stem cells (HSCs) but required for the maturation of megakaryocytes and the differentiation of T- and B-lymphoblasts (Ichikawa et al. 2004). The normal ETO protein, also known as MTG8, is involved in transcriptional regulation because it recruits corepressors such as N-CoR, mSin3A, and SMRT. In the AML1–ETO fusion oncoprotein, a critical lysine residue is acetylated by the transcriptional coactivator p300, an event implicated in AML initiation (Wang et al. 2011). Importantly, AML1–ETO interacts with Smad3 and inhibits its ability to bind to DNA, suppressing TGF-β-Smad signaling (Jakubowiak et al. 2000) (Fig. 8.1). These results support the hypothesis that AML1–ETO drives AML leukemogenesis because it abrogates TGF-β-mediated tumor-suppressive signaling.

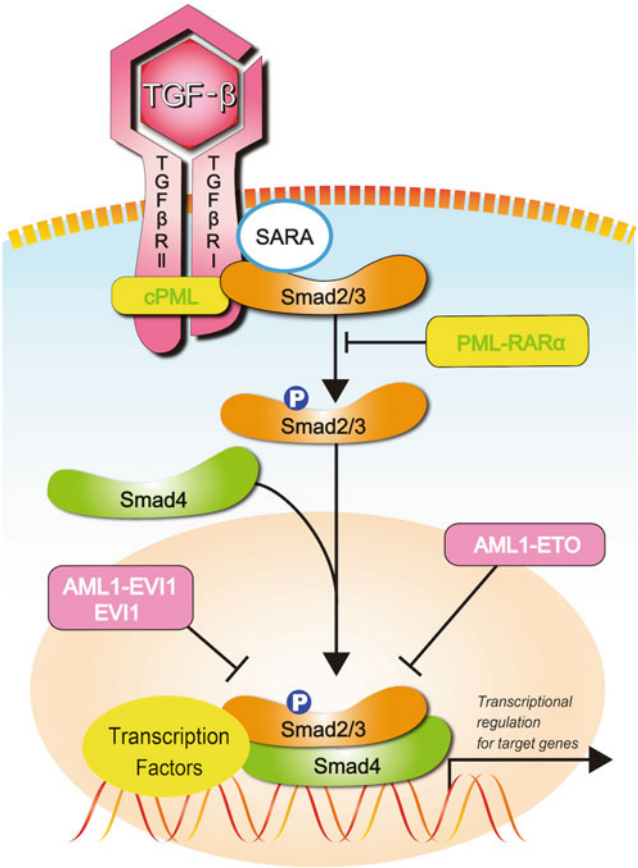

Fig. 8.1 The role of TGF-β signaling in human leukemogenesis. TGF-β signaling is transduced by a cytoplasmic complex containing TGF-β type I receptor (TGF-βRI), TGF-β type II receptor (TGF-βRII), Smad2/3, SARA and cPML. Binding of TGF-β to TGF-βRII triggers the activation of TGF-βRI, which phosphorylates downstream Smad2/3 proteins. The phosphorylated Smad2/3 molecules interact with Smad4, which regulates the expression of genes able to suppress leukemogenesis in the nucleus. The PML–RARα fusion oncoprotein, which causes APL, inhibits cPML function and blocks tumor-suppressive TGF-β signaling. Similarly, the AML1–ETO oncoprotein expressed in AML patients and the AML1–EVI1 oncoprotein or normal EVI1 protein expressed in blast crisis CML patients interact with Smad3 proteins, interfering with their DNA-binding capacity and abrogating TGF-β-mediated growth arrest

8.1.1.2 t(15;17)(q22;q21)-Positive Acute Promyelocytic Leukemia

Acute promyelocytic leukemia (APL, defined as M3 in the FAB classification) is a subtype of AML characterized by the accumulation of abnormal granulated promyelocytes in the bone marrow and peripheral blood. The causal t(15;17)(q24.1;q21.2) translocation generates an oncoprotein (PML–RARα) in which the promyelocytic leukemia (PML) protein is fused to the retinoic acid receptor α (RARα). The normal

PML protein, which is essential for the formation of a dot-like nuclear structure called the "PML nuclear body," has been implicated in transcription, maintenance of genomic integrity, antiviral responses, apoptosis induction, tumor suppression, and stemness. The normal RARα protein belongs to the nuclear receptor superfamily and transduces all-*trans* retinoic acid (ATRA)-mediated transcriptional signaling. APL patients treated with ATRA show PML–RARα degradation and enhanced differentiation of APL cells. Mouse embryonic fibroblasts (MEFs) obtained from *Pml*-deficient mice are resistant to TGF-β-dependent growth arrest, suggesting that PML is involved in the activation of the TGF-β pathway (Lin et al. 2004). Indeed, Lin et al. (2004) reported that a cytoplasmic isoform of PML (cPML) is crucial for TGF-β signaling. The cPML protein participates in the complex containing TGF-βRI, TGF-βRII, Smad2/3, and the "Smad anchor for receptor activation" (SARA) proteins, thereby promoting TGF-β signal transduction. The expression of PML–RARα inhibits cPML function and blocks TGF-β signaling (Fig. 8.1). In *Pml*-deficient MEFs, restoration of cPML function restores normal TGF-β-dependent growth arrest, cellular senescence, and apoptosis. Treatment of APL cell line, NM4, with ATRA increased the interaction between cPML and Smad2/3 and improved their TGF-β responses, implicating PML–RARα-mediated inhibition of TGF-β signaling in APL leukemogenesis (Lin et al. 2004).

8.1.2 Blast Crisis CML

Human CML is a myeloproliferative neoplasm (MPN) that occurs in three phases: (1) an initial chronic phase, (2) an accelerated phase, and (3) finally a blast crisis phase (Ren 2005). The majority of CML patients suffer from the t(9;22)(q34;q11.2) translocation that generates the Philadelphia chromosome and the *BCR–ABL* fusion oncogene, which encodes a constitutively active tyrosine kinase (de Klein et al. 1982; Rowley 1973). Because chronic phase CML cells can still produce functionally normal mature blood cells similar to those in healthy individuals (Fialkow et al. 1977), newly diagnosed CML patients exhibit a massive expansion of myeloid cells that extends for 5–15 years (chronic phase). The acquisition of additional genetic changes and/or epigenetic alterations during this period drives the progression of the disease to the accelerated phase. In the last and lethal blast crisis phase, cells of the myeloid, B-lymphoid, or bi-phenotypic myeloid, and lymphoid mixed lineage are observed (Calabretta and Perrotti 2004).

Blast crisis CML is associated with expression of the AML1–EVI1 fusion oncoprotein generated by the t(3;21)(q26.2;q22.12) translocation. The normal EVI1 protein encodes a zinc-finger transcription factor known to regulate HSC maintenance (Goyama et al. 2008; Yuasa et al. 2005). Excessively high levels of the normal EVI1 protein alone can also cause blast crisis CML (Mitani et al. 1994; Ogawa et al. 1996). Notably, both the normal EVI1 protein and the AML1–EVI1 fusion oncoprotein interact with Smad3 (Fig. 8.1). This association attenuates the DNA-binding activity of Smad3 and thus inhibits the TGF-β signaling pathway (Kurokawa et al. 1998a, b). These findings indicate that loss of TGF-β signaling is implicated in the

progression of CML from the chronic phase to blast crisis and that this interference with TGF-β signaling is due to the interaction of Smad3 with EVI1 or AML1–EVI1.

8.1.3 T-Cell ALL

Letterio and colleagues have examined Smad2/3 proteins in a spectrum of patients with various forms of childhood ALL including T-cell ALL, pre-B-cell ALL, and acute nonlymphoblastic leukemia (ANLL) (Wolfraim et al. 2004). Although Smad2 protein is present at normal levels in leukemic cells of all ALL subtypes examined, Smad3 protein is low in leukemic cells from T-cell ALL patients but not in leukemic cells from pre-B cell ALL or ANLL (Wolfraim et al. 2004). However, Smad3 mRNA has been present at normal levels in leukemic cells from all T-cell ALL patients. Furthermore, no evidence for a deletion or point mutation in any region of the all nine exon of the Smad3 gene (*MADH3*) has been found in any T-cell ALL patients. Nevertheless, although the molecular mechanism responsible for the deficiency of Smad3 protein in these patients has yet to be elucidated, it is tempting to speculate that it might underlie the leukemogenesis of pediatric T-cell ALL. In mice, the loss of a single *Smad3* allele impairs the growth-suppressive effects of TGF-β on normal T-cells (Wolfraim et al. 2004). Moreover, mice harboring a heterozygous deletion of *Smad3* plus homozygous loss of $p27^{Kip1}$ spontaneously develop T-cell leukemia (Wolfraim et al. 2004). These results suggest that a combination of attenuated TGF-β signaling due to *Smad3* deficiency and the homozygous inactivation of $p27^{Kip1}$ might cause pediatric ALL. Indeed, deletions and germline mutations of the *CDKN1B* gene encoding the $p27^{Kip1}$ protein are frequent in pediatric ALL patients (Komuro et al. 1999; Takeuchi et al. 2002).

8.1.4 B-Cell ALL

The t(12;21)(p13.2;q22.12) translocation generating the fusion oncoprotein TEL–AML1 (also known as ETV6–RUNX1) is found in patients with childhood B-cell precursor ALL. Like AML1–EVI1 in AML patients, TEL–AML1 binds to Smad3 and compromises its ability to activate transcription of its target genes (Ford et al. 2009). Murine B-cell progenitor cells expressing TEL–AML1 proliferate more slowly than parental cells but are more resistant to TGF-β-mediated growth suppression (Ford et al. 2009). Importantly, mice expressing a *TEL–AML1* transgene show a greatly expanded number of pre-pro-B cells (Ford et al. 2009). Thus, whereas expression of TEL–AML1 alone is insufficient for the development of full-blown leukemia, this oncoprotein can promote the proliferation of preleukemic cells.

Section summary: The available evidence supports a tumor-suppressive function for TGF-β signaling, at least in differentiated leukemia cells.

8.2 Role of TGF-β-FOXO Signaling in CML Stem Cells

Although TGF-β mediates cytostatic signaling in normal and/or premalignant epithelial cells, it has been clarified that TGF-β promotes their own proliferation, invasion, and metastatic behavior in advanced malignant cells in epithelial-derived tumors (Ikushima and Miyazono 2010; Massagué 2008, 2012; Wakefield and Roberts 2002). In contrast to the case of solid tumor, it has been supposed that suppression of TGF-β signaling pathway is a cause of hematopoietic malignancy, including AML, ALL, and blast crisis CML as described in Sect. 8.1. However, TGF-β signaling and its triggering of the downstream Akt–FOXO pathway paradoxically regulates the self-renewal of CML stem cells and has been linked with their therapeutic resistance. In CML patients with recurrent disease, the reappearance of leukemia is due to the generation of a large number of differentiated CML cells from a rare population of therapy-resistant CML stem cells. Because CML stem cells originate from normal HSCs, it has been suggested that HSCs and CML stem cells share the same molecular mechanisms for regulating the maintenance of their self-renewal ability. In this section of this chapter, we examine the roles of TGF-β and Akt–FOXO signaling in CML stem cells and normal HSCs.

8.2.1 Shared Hallmarks of HSCs and CML Stem Cells

Several lines of evidence implicate normal HSCs as the cell of origin for CML stem cells expressing *BCR–ABL*. (1) Chronic phase CML cells can give rise to multiple lineages of functionally normal mature blood cells (Fialkow et al. 1977), indicating that these CML cells retain normal pluripotent differentiation capacity. (2) The transplantation of murine HSCs engineered to express the human *BCR–ABL* gene induces CML-like MPN in recipient mice (Daley et al. 1990; Elefanty and Cory 1992; Gishizky et al. 1993; Kelliher et al. 1990; Pear et al. 1998). In contrast, the transplantation of murine committed hematopoietic progenitor cells expressing *BCR–ABL* does not induce CML (Huntly et al. 2004). (3) CML stem cells are functionally defined by their ability to induce CML once transplanted into recipient mice. In mice that develop CML-like disease due to retroviral transduction of the *BCR–ABL* gene, CML stem cells can be purified from a rare Lineage$^-$Sca-1$^+$c-Kit$^+$ (LSK) population (i.e., cells that bear the marker profile of normal HSCs) (Hu et al. 2006; Ito et al. 2008; Zhao et al. 2007, 2009). (4) In an inducible *BCR–ABL* transgenic mouse model in which expression of the *BCR–ABL* gene is controlled by a Tet-regulated 3′-enhancer of the murine *SCL* (stem cell leukemia) gene (Huettner et al. 2003; Koschmieder et al. 2005), the CML stem cells driving disease are restricted to populations with the phenotype of long-term HSCs (i.e., CD34$^-$Flt3$^-$LSK or CD150$^+$Flt3$^-$CD48$^-$LSK) (Reynaud et al. 2011; Schemionek et al. 2010; Zhang 2012). (5) In chronic phase CML patients, the CML stem cells responsible for the disease can be isolated from a cell population expressing surface markers associated

with primitive hematopoietic cells (Holyoake et al. 1999, 2001; Pellicano et al. 2009). These observations collectively bolster the hypothesis that HSCs and CML stem cells regulate their self-renewal ability using the same mechanisms. Indeed, several signaling pathways, including those involving Wnt-β-catenin (Scheller et al. 2006; Zhao et al. 2007), PML (Ito et al. 2008), and Hedgehog (Dierks et al. 2008; Zhao et al. 2009), have been implicated in the regulation of stem cell fate during both normal hematopoiesis and CML leukemogenesis. Some researchers have taken these similarities in properties between HSCs and CML stem cells as evidence that CML is a "stem cell disease" (Naka et al. 2010a) and therefore contend that elucidating mechanisms in HSCs will lead to a better understanding of how CML stem cells are perpetuated.

8.2.2 Akt–FOXO Signaling in HSCs

HSCs top the hierarchy of the normal hematopoietic system and are defined by both their ability to reproduce themselves, a property known as self-renewal, and their capacity to give rise to all mature hematopoietic cell lineages. Because HSCs have to sustain hematopoiesis for the life of an individual, the maintenance of HSC quiescence in vivo is crucial for homeostasis. HSC quiescence is adopted within defined "bone marrow niches," which provide a comfortable microenvironment where HSCs can remain in the non-dividing, G0 phase of the cell cycle (Suda et al. 2005). Quiescent HSCs generate only very low levels of reactive oxygen species (ROS), allowing these cells to escape any DNA damage that might be caused by oxidative stress. Genomic integrity is thought to be crucial for the preservation of HSC self-renewal capacity, and it is speculated that dysfunction of genomic integrity causes generation of the Philadelphia chromosome and thereby *BCR–ABL* fusion oncogene.

The self-renewal of normal HSCs also depends on signaling involving the phosphatidylinositol-3-OH kinase (PI3K)–Akt pathway and its downstream targets FOXO1, FOXO3a, FOXO4, and FOXO6, which are members of the Forkhead O (FOXO) subfamily of transcription factors (Greer and Brunet 2005). In the absence of stimulation by growth factors or insulin, FOXOs are present in an active state in a cell's nucleus and freely induce their transcriptional targets. When a growth factor or insulin binds to the appropriate cell surface receptor, PI3K–Akt signaling is activated, and activated Akt directly phosphorylates FOXOs, resulting in their nuclear exclusion and subsequent degradation in the cytoplasm.

FOXO deficiencies have profound effects on HSCs. For example, Tothova et al. reported a marked decrease in HSCs in mice with triple conditional deletions of the *Foxo1*, *Foxo3a*, and *Foxo4* genes (Tothova et al. 2007). The HSCs from these triple *Foxo*-deficient mice not only exhibited a self-renewal defect but also showed elevated ROS. Notably, the expression of superoxide dismutase (*SOD*) genes was impaired in triple *Foxo*-deficient HSCs. In two other studies, Foxo3a was found to be solely responsible for the maintenance of the HSC pool (Miyamoto et al. 2007;

Yalcin et al. 2008). *Foxo3a*$^{-/-}$ HSCs showed reduced expression of the Foxo target genes *SOD2* and *catalase* and exhibited increased ROS, defective self-renewal, and decreased expression of the CDK inhibitors $p27^{Kip1}$ and $p57^{Kip2}$ (Miyamoto et al. 2007; Yalcin et al. 2008). Importantly, $p57^{Kip2}$ and $p27^{Kip1}$ are required for the maintenance and quiescence of HSCs (Matsumoto et al. 2011; Zou et al. 2011). Thus, the FOXO factors are responsible for regulating HSC quiescence, ROS levels and maintenance of self-renewal ability.

The Akt–FOXO pathway in quiescent HSCs within a bone marrow niche is regulated by lipid raft clustering (LRC) (Yamazaki et al. 2009). Lipid raft microdomains, which are patches in the plasma membrane enriched in cholesterol, glycosphingolipids, and glycolipids, control cytokine signaling in murine HSCs. Yamazaki et al. demonstrated that HSCs freshly isolated from mouse bone marrow lacked LRC. Notably, LRC-induced cytokine signaling is essential for the cell cycle reentry of HSCs. Conversely, inhibition of LRC attenuates cytokine signaling, which results in repression of Akt activity followed by Foxo activation. Therefore, the status of the lipid rafts within HSCs in a bone marrow niche fine-tunes Akt–Foxo signaling and regulates HSC quiescence.

8.2.3 TGF-β Signaling in HSCs

Numerous in vitro studies have indicated that TGF-β signaling is involved in regulating mouse and human HSCs (Larsson and Karlsson 2005). Yamazaki et al. clarified that TGF-β signaling controls HSC self-renewal ability by inhibiting LRC and thereby attenuating cytokine signaling (Yamazaki et al. 2009). The inhibition of LRC by TGF-β in HSCs suppressed Akt and induced nuclear localization of Foxo3a. These results link TGF-β signaling with the LRC–PI3K–Akt–Foxo signaling pathway that regulates HSC quiescence in vivo.

While it had been hoped that a genetic approach might reveal the precise role of TGF-β in modulating HSCs in vivo, it has been difficult to carry out the required experiments due to the early lethality and massive inflammatory response in null mutant mice disrupted in the *TGF-β1* (Kulkarni et al. 1993; Shull et al. 1992), *TGF-βRI* (Larsson et al. 2001), or *TGF-βRII* (Oshima et al. 1996) genes. To overcome these difficulties, conditional knockout mice with hematopoietic cell-specific loss of the *TGF-βRI* (*ALK5*) or *TGF-βRII* genes were established using the Cre/LoxP system. Interestingly, while conditional mutants lacking *TGF-βRI* (*ALK5*) had no significant defects in the quiescence and maintenance of HSC pool (Larsson et al. 2003, 2005), *TGF-βRII*-deficient conditional mutants exhibited obvious abnormalities in HSCs (Yamazaki et al. 2011). Similarly, HSCs of conditional *Smad4*-deficient mice showed a significant reduction in self-renewal capacity following transplantation into normal recipient mice (Karlsson et al. 2007). Interestingly, although Smad4 plays an integral role in both the TGF-β and bone morphogenetic protein (BMP) pathways, studies of conditional mutant mice disrupted in the *Smad1* and *Smad5* genes revealed that canonical BMP signaling is

dispensable for HSC maintenance (Singbrant et al. 2010). Thus, it is the TGF-β-Smad pathway that is required for HSC maintenance in vivo (Yamazaki et al. 2011). However, conditional disruption of the *BMP receptor type 1A* (*Bmpr1a*) gene in mice results in an increased number of osteoblasts, which expand the size of the HSC bone marrow niches. This expansion in turn facilitates the enlargement of the HSC pool, indicating that BMP signaling is indirectly involved in the control of HSC numbers (Zhang et al. 2003).

HSCs themselves produce TGF-β in a latent form but are not able to process it into its active structure. Yamazaki et al. have shown that bone marrow glial cells expressing glial fibrillary acidic protein (GFAP) regulate the maintenance of quiescent HSCs by activating their latent TGF-β (Yamazaki et al. 2011). These GFAP-expressing glial cells have been identified as non-myelinating Schwann cells that ensheath sympathetic nerves in the bone marrow. Importantly, when the number of active TGF-β-producing cells is reduced via autonomic nerve denervation, HSCs are missing from the bone marrow (Yamazaki et al. 2011). Taken together, these results indicate that the signaling of TGF-β family members within the bone marrow niche has profound regulatory effects on HSC properties.

8.2.4 Opposing Roles of FOXOs in CML Non-stem Cells vs. CML Stem Cells

Given that CML stem cells originate from normal HSCs, it has been of great interest to determine whether CML stem cells conserve the regulatory mechanisms found in normal HSCs. Studies using CML cell lines have shown that, in CML non-stem cells, activated FOXOs can induce apoptosis or cell cycle arrest (Essafi et al. 2005; Ghaffari et al. 2003; Komatsu et al. 2003). The BCR–ABL fusion protein in CML cells drives strong Akt activation that leads to nuclear export of Foxo3a and forceful repression of Foxo3a functions (Essafi et al. 2005; Ghaffari et al. 2003; Komatsu et al. 2003). When TKIs block BCR–ABL and suppress Akt activity, Foxo3a remains activated and triggers apoptosis or cell cycle arrest (Fig. 8.2, left). Based on these findings, it has been believed that FOXOs are suppressors of CML leukemogenesis. However, Komatsu et al. insightfully reported several years ago that Foxo3a is involved in the acquisition of dormancy in CML cell lines exposed to imatinib (Komatsu et al. 2003). Indeed, when we investigated whether Foxo3a was similarly implicated in the maintenance of CML stem cells using a mouse CML model, we found that, unlike CML non-stem cells, CML stem cells exhibit decreased Akt phosphorylation and increased nuclear localization of Foxo3a in vivo (Naka et al. 2010b). In line with this observation, Lee et al. reported that Foxo3a was localized in the nucleus of a primitive $CD150^{+}CD48^{-}CD41^{-}$LSK population in a *Pten-deficient* MPN mouse model (Lee et al. 2010). Thus, it appears that the maintenance of the rare CML stem cell population is controlled by different regulators than those governing the majority of CML non-stem cell population.

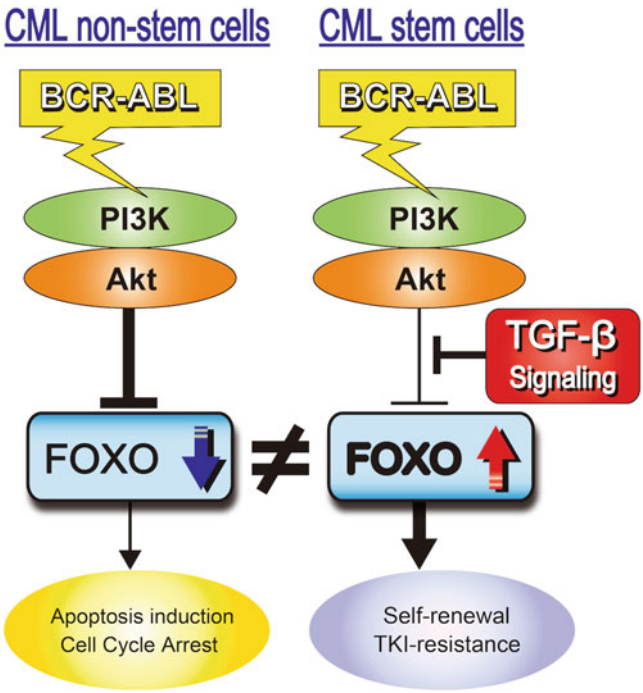

Fig. 8.2 Opposing roles of the TGF-β-FOXO axis in CML stem cells vs. CML non-stem cells. FOXO transcription factor is generally thought to suppress tumorigenesis because activated FOXO induces the apoptosis or cell cycle arrest. In CML non-stem cells (*left*), FOXO functions are suppressed by BCR–ABL, which drives the activation of PI3K–Akt signaling pathway. In CML non-stem cells treated with TKIs, BCR–ABL activity is blocked, Akt activity is suppressed, and FOXOs remain activated. Differentiated leukemic cells are thus reduced in number. Paradoxically, in CML stem cells (*right*), the TGF-β-FOXO axis is tumor-promoting. BCR–ABL-triggered activation of the PI3K–Akt signaling pathway is inhibited by TGF-β signaling. As a consequence, FOXOs remain active and drive the self-renewal and TKI resistance of CML stem cells. Because BCR–ABL does not function in CML stem cells, TKIs have no effect on them and cannot eradicate them. Thus, it is possible that the therapeutic suppression of the TGF-β-FOXO axis might be a novel strategy for the eradication of CML stem cells and thus the inhibition of leukemia recurrence that is frequently lethal

To clarify the functional role of Foxo3a in CML stem cells, we transplanted *Foxo3a*$^{+/+}$ and *Foxo3a*$^{-/-}$ CML stem cells into recipient mice (Naka et al. 2010b). Both groups of recipients died of CML-like MPN with similar kinetics, indicating that Foxo3a is dispensable for the initiation of CML. However, serial transplantation of *Foxo3a*$^{+/+}$ and *Foxo3a*$^{-/-}$ CML stem cells revealed that the ability of CML stem cells to perpetuate disease in vivo is significantly decreased by *Foxo3a* deficiency. Moreover, recipients that received *Foxo3a*$^{+/+}$ CML stem cells developed both ALL and CML, demonstrating that these CML stem cells are able to generate malignancies in multiple hematopoietic lineages. In contrast, recipients transplanted with *Foxo3a*$^{-/-}$ CML stem cells developed neither ALL nor CML. Thus, Foxo3a

sustains the long-term maintenance of multi-lineage leukemia-initiating potential in CML stem cells. These findings underpin the paradoxical concept that FOXOs play opposite roles in the survival of CML stem cells vs. CML non-stem cells (Fig. 8.2).

The counterintuitive idea that FOXOs are active in leukemia stem cells can be extended to AML. In AML patients, Foxo signaling is activated in primitive $CD34^{+}lineage^{low}$ bone marrow cells (Sykes et al. 2011). Similarly, in a mouse AML model harboring the human *MLL-AF9* leukemic gene, mice transplanted with leukemia cells triply deficient for *Foxo1/3/4* display a longer latency to disease coupled with a decreased frequency of leukemia-initiating cells (leukemic-granulocyte-macrophage progenitor cells; L-GMP) compared to mice transplanted with leukemia cells possessing intact Foxo alleles (Sykes et al. 2011). Taken together, these results indicate that FOXOs are involved in the maintenance of leukemic stem cells in both CML and AML.

8.2.5 FOXO is Crucial for TKI Resistance in CML Stem Cells

The discovery of TKIs such as imatinib mesylate, nilotinib, and dasatinib that could directly inhibit the Abl kinase activity of the BCR–ABL oncoprotein was a dramatic breakthrough for the treatment of CML patients. However, although 95 % of imatinib-treated chronic phase CML patients survive for over 5 years (Druker et al. 2006), resistance to or intolerance of these drugs inevitably develops (Bhatia et al. 2003; Corbin et al. 2011; Cortes et al. 2004; Goldman and Gordon 2006; Savona and Talpaz 2008). Recent studies have demonstrated that TKIs are potent killers of differentiated leukemic cells, which are proliferating, but cannot deplete primitive CML stem cells, which are quiescent (Copland et al. 2006; Graham et al. 2002; Jorgensen et al. 2007; Konig et al. 2008a, b, c). It is possible that quiescent CML stem cells remaining after a first round of TKI treatment acquire mutations of the *BCR–ABL* gene itself. Such mutations would be manifested as TKI resistance in CML patients treated with a second round of TKI therapy (Gorre et al. 2001; Roumiantsev et al. 2002). Thus, many oncologists believe that TKI inhibitors coupled with novel therapeutics capable of eradicating quiescent CML stem cells may prevent disease recurrence and greatly benefit CML patients (Kavalerchik et al. 2008).

Because FOXOs play an essential role in the long-term maintenance of CML stem cells, we examined whether FOXOs also contribute to their TKI resistance. Although mice transplanted with *Foxo3a*$^{+/+}$ or *Foxo3a*$^{-/-}$ CML stem cells showed similar rates of lethality, the survival of mice that received *Foxo*-deficient CML stem cells and were treated with imatinib was significantly prolonged (Naka et al. 2010b). Furthermore, after imatinib administration, the number of residual CML stem cells present in mice transplanted with *Foxo3a*$^{-/-}$ CML stem cells was less than in mice transplanted with *Foxo3a*$^{+/+}$ CML stem cells. Interestingly, Muschen and colleagues have shown that the *BCL-6* proto-oncogene, which is a downstream effector of FOXO, is essential for both the maintenance of CML stem cells and the TKI resistance of ALL cells positive for the Philadelphia chromosome (Duy et al. 2011;

Hurtz et al. 2011). Therefore, therapeutics targeting FOXOs might help to eradicate CML stem cells in CML patients and render TKI-resistant cells in Ph⁺ALL patients susceptible to treatment.

8.2.6 *TGF-β Regulates FOXO Activity and the Self-Renewal Ability of CML Stem Cells*

Because TGF-β signaling regulates the Akt–FOXO pathway in normal HSCs, we investigated the impact of the TGF-β inhibitor Ly364947 on CML stem cell functions in vivo (Naka et al. 2010b). The administration of Ly364947 alone to CML-affected mice leads to Akt activation and decreased nuclear Foxo3a in CML stem cells, demonstrating that TGF-β is a critical regulator of Akt and Foxo in these cells in vivo. Notably, although administration of Ly364947 alone does not extend the survival of CML-affected mice, the administration of Ly364947 combined with imatinib treatment significantly reduces recipient lethality, CML stem cell frequency, and CML infiltration in the lung (Naka et al. 2010b). These findings indicate that the TGF-β-FOXO axis plays a critical role in the maintenance of TKI-resistant CML stem cells.

The sources of TGF-β that could support CML stem cell survival in vivo are under intense investigation. For normal HSCs, the activation of latent TGF-β is mediated by GFAP-expressing glial cells in the bone marrow (Yamazaki et al. 2011), making these cells important candidates for the source of TGF-β also maintaining the CML stem cell niche. In addition, Yokota et al. have demonstrated that osteoclasts are involved in the maintenance of dormant CML cells in the bone marrow through their release of TGF-β (Yokota et al. 2010). Recently, it was reported that an individual's genotype at the *killer immunoglobulin-like receptor* (*KIR*) genes is predictive of a complete cytogenetic response (CCyR) and survival rate in CML patients who are treated with imatinib (Marin et al. 2012). KIRs are expressed by natural killer (NK) cells, which are cells of the innate immune response. The physiological functions of NK cells, which include cytotoxicity and cytokine production, are controlled by a balance of inhibitory and activatory signals from cell surface receptors such as the KIRs. Significantly, NK cells are increased in number in CML patients treated with TKIs (Kreutzman et al. 2010; Yong et al. 2009). Marin et al. found that patients carrying the activatory receptor *KIR2DS1* have a significantly lower probability of achieving CCyR within 2 years of TKI treatment compared with patients who are *KIR2DS1*-negative (Marin et al. 2012). Importantly, NK cells expressing *KIR2DS1* secrete TGF-β (Ghio et al. 2009), implicating these cells as a possible source of the TGF-β driving the TKI resistance of CML stem cells.

Because TGF-β signaling is responsible for the maintenance of TKI-resistant CML stem cells in vivo, the suppression of TGF-β signaling so as to eradicate CML stem cells is a plausible therapeutic strategy. Although TGF-β-Foxo signaling is essential for the maintenance of both normal HSCs and CML stem cells, inhibition of TGF-β has a much more dramatic effect on CML stem cells than on normal HSCs. Notably, in the presence of imatinib, the treatment in vitro of

primitive $CD34^+$ human CML cells with TGF-β inhibitors attenuates their viability (Moller et al. 2007; Naka et al. 2010b). These results suggest that judicious inhibition of TGF-β-FOXO signaling may lead to efficient eradication of residual CML stem cells.

Section Summary: Because the activation of TGF-β signaling may be implicated in CML leukemogenesis, the application of TGF-β inhibitors to the treatment of CML patients is a promising therapeutic avenue. If detrimental effects on normal hematopoietic cells can be avoided, it is possible that the inhibition of TGF-β signaling might specifically deplete CML stem cells in patients with chronic phase CML and that a combination of a TGF-β inhibitor with TKIs might be effective in removing residual CML stem cells that survive therapy with TKI alone.

8.3 Conclusion

In this chapter, we have reviewed the multifaceted role of TGF-β signaling in leukemogenesis. As investigators have believed for many years, TGF-β signaling in normal HSCs helps to suppress the rise of several types of differentiated leukemia cells, including those present in AML, blast crisis CML, and pediatric ALL. Therefore, it is important to elucidate how the chromosomal translocations associated with these disorders generate oncoproteins that can inhibit TGF-β-mediated growth suppression. It is also important to recognize that TGF-β-FOXO signaling has the opposite effect on CML stem cells and is responsible for their survival and TKI resistance. Moreover, like normal HSCs, CML stem cells are quiescent, stress-resistant, and maintain their self-renewal ability within a niche. Thus, for the successful development of new small molecule drugs capable of eliminating CML stem cells, it may be advantageous to target the TGF-β signaling network (including elements of the Akt–FOXO pathway) and the niche cells that support it. The ultimate goal for this field is to develop novel therapeutics that can eradicate leukemia stem cells, thereby forestalling disease recurrence and providing concrete benefits for patients.

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Chapter 9
TGF-β in Skin Cancer and Fibrosis

Gangwen Han, Zheyi Han, and Xiao-Jing Wang

Abstract Among all three isoforms of the TGF-β ligand, TGF-β1 is the predominant isoform in the skin. In normal skin, canonical TGF-β signaling components, i.e., TGF-β receptors and signaling Smads, are broadly and highly expressed, whereas TGF-β ligands are expressed at very low levels. These expression patterns determine that the TGF-β signaling input to the skin is low under normal conditions but high once TGF-β ligands are upregulated under disease conditions. TGF-β1 is a potent growth inhibitor of epidermal keratinocytes, which dictates its tumor suppressive effect in early stages of skin cancer. However, cancer cells lose TGF-β-induced growth inhibition at late stages, and TGF-β-induced angiogenesis and skin inflammation create an environment favorable for skin cancer progression and metastasis. In fibrotic skin diseases, TGF-β plays a key role in activating fibroblast proliferation and stimulating the production of extracellular matrix proteins. Also, TGF-β affects many aspects of skin wound healing and in turn influences cutaneous scarring after skin damage. Fully understanding the mechanisms of TGF-β in the pathogenesis of skin cancer and fibrotic diseases will help design novel strategies in treating skin diseases.

Keywords Cutaneous scarring • Smad • Squamous cell carcinoma (SCC) • Systemic sclerosis (SSc) • TGF-β • Wound healing

9.1 Introduction

Transforming growth factor-β (TGF-β) is a ubiquitous and multifunctional cytokine that regulates a variety of events in cells and tissues, including cell proliferation, differentiation, migration, angiogenesis, inflammation, and immune surveillance.

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The function of TGF-β is largely dependent on its targeted cells and is influenced by other molecules and signaling. Thus, the in vivo role of TGF-β is complicated and context dependent. The skin is histologically divided into two parts: the epidermis and the dermis. Keratinocytes in the epidermis and fibroblasts in the dermis represent two major cell types showing opposite effects in response to TGF-β: TGF-β inhibits keratinocytes but promotes fibroblast proliferation (Anzano et al. 1982; Coffey et al. 1988; Shipley et al. 1986). Among the three TGF-β isoforms, TGF-β1 is the predominant isoform in most tissue types, including the skin (Frank et al. 1996; Quan et al. 2002; Wang 2001), and is expressed at a very low level in normal skin but elevated in skin diseases and cancer (Han et al. 2005; Quan et al. 2002; Querfeld et al. 1999). The role of TGF-β in the pathogenesis of skin cancer and fibrotic diseases has been extensively studied. In addition to the direct effect of TGF-β on keratinocytes and fibroblasts, TGF-β-mediated inflammation, angiogenesis, and regulation of immune cells have additional impacts on the development of skin cancer and fibrosis (Li et al. 2006; Martin 1997; Seifert and Mrowietz 2009; Wei et al. 2011). Particularly, recent studies on the receptors and Smads, components of TGF-β signaling in transgenic/knockout mouse models further advanced our understanding of the roles of TGF-β signaling in skin cancer and fibrotic diseases (Han and Wang 2011; Lakos et al. 2004; Sonnylal et al. 2007; Wei et al. 2011). This chapter will focus on the role of TGF-β signaling in the pathogenesis of non-melanoma skin cancer and skin fibrotic disorders, the latter including systemic sclerosis (SSc), hypertrophic scarring, and keloid scarring. Because hypertrophic and keloid scarring are consequences of wound healing affected by TGF-β during the healing process, the role of TGF-β signaling in cutaneous wound healing is also discussed.

9.2 TGF-β Signaling in Non-melanoma Skin Cancer

Non-melanoma skin squamous cell carcinoma (SCC) is the most common human cancer developed from epidermal keratinocytes. Genetic mutations and continuous proliferation of mutant keratinocytes are the prerequisites for SCC development. Studies indicate that angiogenesis, inflammation, and alterations in immune response in the stroma significantly influence the development of skin cancer and metastasis. Many molecules and signaling pathways have been implicated in the pathogenesis of SCC. The role of TGF-β and its downstream signaling components have been extensively investigated in SCC. The TGF-β signaling pathway consists of three components: (1) Ligands, i.e., TGF-β1, 2, and 3. (2) Secreted ligands function through two serine/threonine kinase receptors, TGF-β receptor type I (TGF-βRI) and type II (TGF-βRII), both of which are necessary for signal transduction. TGF-βRII receptor directly binds to ligands and interacts with TGF-βRI. (3) Intracellular downstream mediators, i.e., Smads, are phosphorylated and activated by TGF-βRI. To date, many components of TGF-β signaling have been studied for their differential roles in normal keratinocytes, SCC cells and tumor stromal cells. This chapter will give a brief review over these differential roles of TGF-β signaling in skin carcinogenesis.

9.2.1 *TGF-β Inhibits Tumor Formation of* ras *Transformed Keratinocytes*

TGF-β was initially identified as a growth factor stimulating the growth of normal rat kidney fibroblasts (Anzano et al. 1982; Sporn 1999, 2006). Soon, however, TGF-β was found to inhibit mink lung epithelial cell growth (Tucker et al. 1984). Thus, the diversity of TGF-β's functions on different cell types was quickly recognized (Roberts et al. 1985). In particular, TGF-β inhibits the growth of normal human keratinocytes but can lose its growth inhibitory responses as it happens in the skin cancer cell line SCC-25 due to the possibility that tumor cells lack TGF-β receptors (Coffey et al. 1988; Shipley et al. 1986). However, tumor development in normal keratinocytes requires an initial oncogenic mutation. The *Ras* mutation in keratinocytes is considered an important initiating factor for papilloma development in mouse skin (Roop et al. 1986) and was identified in some human skin cancers (Dlugosz et al. 2002). Thus, *Ras*-transfected keratinocytes have been used to study the role of TGF-β signaling in skin carcinogenesis. In vitro studies showed that *v-ras*^{Ha} transfected normal mouse keratinocytes increased basal expression and secretion of TGF-β1, and grafting *v-ras*^{Ha} transfected normal keratinocytes onto nude mice formed well-differentiated papillomas with increased expression of TGF-β1 protein in the basal and spinous layers of papillomas (Glick et al. 1991, 1994). However, *v-ras*^{Ha} transfected TGF-β1 null mouse keratinocytes grafted onto nude mice progressed rapidly to multifocal SCC. Tumor proliferation was also elevated in grafts initiated from *v-ras*^{Ha} transfected TGF-β1 null keratinocytes compared to cells with *v-ras*^{Ha} transfected wild-type keratinocytes. These studies indicated that TGF-β provides a tumor-suppressing function in skin carcinogenesis (Glick et al. 1991, 1994). Similarly, transient inactivation of TGF-β signaling by infecting with a dominant-negative TGF-βRII causes chromosome instability. These phenomena, observed in keratinocytes with TGF-β1 deletion or expressing dominant-negative TGF-βRII, could be suppressed by exogenous TGF-β1 (Glick et al. 1999). In the TGF-β signaling pathway, Smad3 is a critical factor mediating TGF-β signaling to its target genes (Millet and Zhang 2007). Smad3 null keratinocytes transduced with the *v-ras*^{Ha} gene lost their growth inhibitory response to TGF-β. Nude mice with *v-ras*^{Ha}-transduced Smad3 null keratinocyte grafts developed more papillomas and progressed to SCC with a higher frequency than nude mice with *v-ras*^{Ha}-transduced Smad3 wild-type keratinocyte grafts (Vijayachandra et al. 2003). Therefore, data based on in vitro models of keratinocyte transformation studies demonstrated that TGF-β signaling inhibits keratinocyte proliferation and defects in TGF-β signaling accelerate tumor progression in multistage mouse carcinogenesis (Glick et al. 1999). HaCaT is a spontaneously immortalized normal human keratinocyte cell line (Boukamp et al. 1988). The cell line keeps most characteristics of normal keratinocytes and remains non-tumorigenic after long-term culture and passage. HaCaT cell became tumorigenic after transducing with a *ras* oncogene (Fusenig and Boukamp 1998; Gold et al. 2000), Within established *ras-transfected* HaCaT cells, benign and malignant clones have been characterized

based on their behaviors in forming tumor after transplantation into nude mice (Fusenig and Boukamp 1998). HaCaT-*ras* cells with stable transduction of dominant-negative TGF-βRII lose differentiation and form metastatic SCC in vivo (Ganapathy et al. 2010). In contrast to the growth inhibitory effect of TGF-β on normal keratinocytes, HaCaT-*ras* cells with high TGF-β1 production in cultured conditions have increased tumorigenesis in vivo, and transducing TGF-β1 or TGF-β2 to the benign HaCaT-*ras* clone increases tumor formation, invasion, and metastasis in nude mice (Davies et al. 2012). Furthermore, the HaCaT-*ras* cells producing more TGF-β still retain the response to TGF-β-induced growth inhibition in vitro (Davies et al. 2012), suggesting that the growth inhibitory effect of TGF-β1 is insufficient for tumor suppression in vivo.

9.2.2 In Vivo Expression of TGF-β1 in the Skin Inhibits Benign Tumor Formation and Promotes Malignant Conversion in Skin Carcinogenesis

The two-stage chemical carcinogenesis model is a well-established experimental system to study the mechanism of skin tumor development in vivo (Filler et al. 2007; Yuspa 1986). In this model, skin tumor is first initiated by exposing the skin to carcinogens such as the mutagen dimethylbenzanthracene (DMBA), which can cause mutation in the oncogene *Ha-ras* (Fujiki et al. 1989); second, tumor formation is promoted by treatments of a tumor promoter, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Some benign tumors (primarily papillomas) can convert to malignant SCCs after acquiring additional spontaneous genetic mutations (Akhurst and Balmain 1999). In wild-type mice, TGF-β1 mRNA expression is quickly induced by TPA in suprabasal cells of the epidermis (Akhurst et al. 1988). Chemically induced papillomas and carcinomas have elevated levels of TGF-β1 mRNA in the keratinocyte compartment (Fowlis et al. 1992; Patamalai et al. 1994). To investigate the role of TGF-β in skin homeostasis and carcinogenesis in vivo, keratinocyte-specific TGF-β transgenic mice were developed. Transgenic mouse models that target TGF-β1 to epidermal keratinocytes using different keratin promoters demonstrate that functions of TGF-β1 in skin development and epidermal keratinocyte proliferation depending on the location of TGF-β1 expression in different epidermal layers. When TGF-β1 was targeted to suprabasal layers of the epidermis driven by keratin 1 (K1) promoter (K1.TGF-β1), transgenic mice died at early neonatal stages (Sellheyer et al. 1993) due to the inhibition of keratinocyte proliferation. In contrast, mice overexpressing TGF-β1 driven by keratin 6 or keratin 10 promoters (K6.TGF-β1 or K10.TGF-β mice) lived to adulthood with no significant histological changes other than resistance to TPA-induced epidermal hyperplasia (Cui et al. 1995; Fowlis et al. 1996). However, study of long-term chemical carcinogenesis on K6.TGF-β1 and K10.TGF-β1 transgenic mice led to the conclusion that TGF-β1 has a biphasic effect on skin carcinogenesis: inhibiting tumorigenesis at an early stage but promoting malignant tumor conversion and rapid metastasis at a later stage (Cui et al. 1996). Using gene-switch-TGF-β1 transgenic mice to induce TGF-β

1 transgene expression in the skin at specific stages of chemical carcinogenesis, we found that TGF-β1 transgene induction at early stage inhibited tumor formation but TGF-β1 induction in papillomas significantly promoted tumor conversion from benign to malignant with increased metastasis (Weeks et al. 2001). Moreover, TGF-β transgenic papillomas exhibited down-regulation of TGF-β receptors and their signal transducer Smads, loss of the invasion suppressor E-cadherin/catenin complex in the cell membrane, elevated expression of matrix metalloproteinases and increased angiogenesis. Thus, although down regulation of TGF-β signaling components in tumor epithelia abolishes TGF-β-induced tumor cell growth inhibition, tumor stroma, which has intact TGF-β signaling components, respond to increased TGF-β ligand to promote tumor metastasis (Weeks et al. 2001).

9.2.3 In Vivo Abrogation of TGF-β Receptors in Skin Promotes Tumor Development and Metastasis

Mice with germline TGF-βRII deletion are embryonic lethal around 10.5 days of gestation (Oshima et al. 1996). Interruption of TGF-βRII function by either overexpressing dominant-negative TGF-βRII (ΔβRII) receptors or conditional deletion of TGF-βRII in specific epidermis has been utilized to study the role of TGF-β receptors in skin homeostasis and carcinogenesis (Amendt et al. 1998; Go et al. 1999; Guasch et al. 2007). Transgenic mice expressing ΔβRII in suprabasal keratinocytes driven by truncated mouse loricrin promoter (ML.ΔβRII) develop hyperproliferative skin conditions in the early neonatal stage but normalize in adulthood. In vitro cultured primary keratinocytes from these mice demonstrated resistance to exogenous TGF-β1-induced growth inhibition (Wang et al. 1997). When these transgenic mice were subjected to the two-stage chemical carcinogenesis study, they developed tumors earlier with a larger number of benign papillomas and higher frequencies of malignant cancer formation and metastasis compared to wild-type mice (Go et al. 1999). Analysis showed that ML.ΔβRII tumors had increased expressions of vascular endothelial growth factor (VEGF), a pro-angiogenesis factor, and decreased expression of thrombospondin-1, an angiogenesis inhibitor. Both factors are attributed to the increased angiogenesis found in tumors and malignant carcinoma progression and metastasis. Increased angiogenesis correlated with elevated endogenous TGF-β1 in ML.ΔβRII tumors (Go et al. 1999). Similarly, when ΔβRII was expressed in basal keratinocytes in mouse skin targeted by the keratin 5 promoter, the transgenic mice also have accelerated skin tumor development and malignancy transformation in chemical carcinogenesis (Amendt et al. 1998) compared to wild-type mice. Moreover, keratinocyte-specific TGF-βRII knockout mice in which deletion of TGF-βRII in stratified epithelia driven by the keratin 14 promoter results in spontaneous SCC formation in the transitional area between the stratified squamous epithelium of the skin and the mucosal epithelium, e.g. the anal and genital regions but not in mouse back skin (Guasch et al. 2007). Further analyses revealed that in comparison with mouse back skin, the epithelium of the transitional zone in the anus naturally showed enhanced *Ras*-MAPK signaling, locally increased inflammation

and aberrant differentiation, which favor to tumorigenesis in the anal and genital regions. In aged mice with TGF-βRII deletion, reduced apoptosis and high epidermal proliferation due to blocking of TGF-β signaling eventually caused anal and genital SCC development. Tumor formation in these regions did not happen in young mice when enhanced proliferation, as a result of TGF-βRII deletion, was balanced with increased apoptosis (Guasch et al. 2007). Furthermore, when DMBA alone was applied to TGF-βRII knockout mice skin or grafting of *Ha-Ras*-infected TGF-βRII knockout keratinocytes onto nude mice, mice quickly developed invasive/metastatic SCC (Guasch et al. 2007). Therefore, the loss of TGF-β function in epidermal keratinocytes promotes skin carcinogenesis, but development of malignant tumors requires additional initiation factors (Amendt et al. 1998; Go et al. 1999; Guasch et al. 2007). Human malignant skin cancer frequently exhibits overexpression of TGF-β1 but reduced expression of TGF-βRII. To better understand how this combination affects cancer prognosis, we studied skin carcinogenesis using the chemical carcinogenesis protocol in inducible TGF-β transgenic mice with and without functional TGF-β receptors. In this study, TGF-β1/ΔβRII compound mice were generated; these mice stably express ΔβRII, which inhibits cell binding to the TGF-β ligand, but TGF-β1 can be induced at specific stages of carcinogenesis. When TGF-β1 expression was induced in the papilloma stage, mice developed malignant SCC at a high frequency, with more metastasis to the lymph nodes and lung. Overall, 30 % of tumors in mice overexpressing TGF-β1 exhibited spindle cell carcinoma (SPCC). TGF-β1 induction in the papilloma with inactivation of TGF-βRII function in the TGF-β/ΔβRII compound mice also developed more malignant SCC and metastasis. However, SPCC was rarely observed in TGF-β1/ΔβRII mice. Consistently, TGF-β1-induced SCCs exhibited loss of E-cadherin, an EMT marker. This phenomenon was not common in ΔβRII or TGF-β1/ΔβRII compound mice. Further analysis showed that TGF-β1-mediated SCCs had significant levels of Hey1 and Jag1, while upregulation of Erk1, JNK1, and RhoA/Rac1 was also observed in all TGF-β1 and ΔβRII mice. Therefore, TGF-β1-induced EMT and metastasis can be uncoupled. TGF-β1 mediates EMT via the activation of Notch signaling and requires intact TGF-β signaling components, whereas increased angiogenesis and matrix metalloproteinase (MMP) caused by TGF-β-induced MAPK and Rho/Rac activation via a Smad-independent mechanism largely contribute to cancer metastasis (Han et al. 2005). This study highlights an important fact that EMT cannot always be used to predict the metastatic potential.

9.2.4 Differential Roles of TGF-β Signaling Smads in Skin Cancer

The discovery and study of Smad family members mediating TGF-β signaling has significantly improved the understanding of the TGF-β signaling pathway's multiple biological functions. Eight isoforms of Smads have been found in mammals and are generally classified into three subtypes: R-Smads (Smad1, 2, 3, 5, 8), Co-Smad (Smad4), and I-Smads (Smad6, 7) (Derynck and Zhang 1996; Massague 1996).

Germline deletions of Smad1, Smad2, Smad4, Smad5, and Smad7 in mice result in embryonic death (Chang et al. 1999; Heyer et al. 1999; Kleiter et al. 2010; Nomura and Li 1998; Sirard et al. 1998; Tremblay et al. 2001; Waldrip et al. 1998; Weinstein et al. 1998; Yang et al. 1998). Smad3 knockout mice survive to adulthood (Datto et al. 1999; Yang et al. 1999; Zhu et al. 1998). To overcome embryonic lethality caused by knocking out individual Smads, conditional knockout mice targeting Smad deletion to keratinocytes were generated to study the role of Smads in skin carcinogenesis (Bornstein et al. 2007). So far, the potential roles of TGF-β signaling Smads, i.e., Smad2, Smad3, and Smad4, in skin carcinogenesis have been investigated, but the role of other Smads in skin tumors remains to be clarified. Thus, the current discussion will be focused on the role of TGF-β signaling Smads in skin cancer.

9.2.4.1 The Role of Smad2 in Skin Carcinogenesis

Loss or reduction of Smad2 expression occurs in several human SCCs, including genital SCCs, head and neck SCCs and cervical SCCs (Han and Wang 2011). Immunostaining has shown Smad2 protein reduction/loss in 70 % of human skin cancers. Smad2 loss is especially high in poorly differentiated SCCs (Hoot et al. 2008). Although Smad2 mutation was not frequent in human SCCs (Han and Wang 2011), we found that approximately 67 % of poorly differentiated human skin SCCs have loss of heterozygosity (LOH) at the Smad2 locus, possibly due to accumulated ultraviolet radiation-induced genomic damage (Hoot et al. 2008). Since germline homozygous Smad2 deletion in mice results in embryonic lethality (Heyer et al. 1999; Nomura and Li 1998; Waldrip et al. 1998; Weinstein et al. 1998), the role of Smad2 in skin carcinogenesis was first investigated in Smad2 heterozygous mice (Smad2+/−) because these mice lived a normal lifespan without spontaneous tumor development. Smad2+/− mice developed a greater number of less-differentiated tumors compared to wild-type control mice when exposed to the two-stage chemical carcinogenesis protocol (Tannehill-Gregg et al. 2004). In our established conditional Smad2 knockout mice that target Smad2 deletions to keratinocytes using the keratin 14 (K14) promoter, we found Smad2 deletion did not develop spontaneous skin tumors throughout 18 months of observation (Hoot et al. 2008). Moreover, even in the presence of a DMBA-induced *H-ras* mutation, a genetic alteration mimicking early stage human skin cancer, mice with epidermal Smad2 deletion still failed to develop skin tumors without TPA promotion. Thus, Smad2 loss alone is insufficient to promote initiated cells to develop into cancer. Similar to the observation in Smad2+/− mice (Tannehill-Gregg et al. 2004), when the two-stage chemical carcinogenesis protocol was applied to mice with epidermal Smad2 loss, mice developed skin tumors early with increased numbers, and accelerated malignant conversion compared to wild-type mice (Hoot et al. 2008). Histological analysis showed that Smad2 loss induced papillomas to undergo early EMT, and about 25 % malignant tumors in conditional Smad2 knockout mice were classified as SPCC, which are rare in wild-type mice. Moreover, Smad2 deficient tumors have significantly increased angiogenesis in the stroma. Mechanism analyses revealed that levels of TGF-β1, TGF-β signaling, Smad3/Smad4, and VEGF were similar between

Smad2 deletion tumors and wild-type tumors. Proliferation and apoptosis in tumors have a very limited effect from Smad2 deletion. However, Smad2 deletion significantly increased Snail and hepatocyte growth factor (HGF) expression in keratinocytes and tumor epithelial cells. Further analysis identified that Smad2 loss causes Snail and HGF upregulation via loss of Smad2-mediated transcriptional repression and enhanced Smad3/4-mediated transactivation for Snail and HGF in keratinocytes and tumor cells (Hoot et al. 2008; Hoot et al. 2010). These results provide potential new targets for treating skin cancer.

9.2.4.2 The Role of Smad3 in Skin Carcinogenesis

Smad3 acts as a major mediator for the TGF-β signaling pathway (Millet and Zhang 2007; Nakao et al. 1997). With respect to the role of Smad3 in SCC, Smad3 null keratinocytes transduced with *v-ras*^{Ha} exhibited significant reduction of TGF-β-induced cell growth arrest and increased tumorigenesis after grafting onto nude mice. Overexpression of Smad3 in wild-type keratinocytes by infection with adenoviral-Smad3 induced keratinocyte growth arrest and senescence. All of the changes observed in Smad3 null keratinocytes cannot be amended by transfecting Smad2 and Smad4 (Bae et al. 2009; Vijayachandra et al. 2003). In contrast to the findings in Smad3 null keratinocytes and keratinocyte grafting bioassays, a carcinogenesis study in Smad3 knockout mice has shown that Smad3 is required for tumor formation in the two-stage carcinogenesis model. Germline Smad3 knockout mice did not develop spontaneous skin tumors after long-term observation (Datto et al. 1999; Yang et al. 1999; Zhu et al. 1998). Interestingly, both homozygous and heterozygous Smad3 knockout mice have shown resistance to chemically induced skin carcinogenesis (Li et al. 2004a; Tannehill-Gregg et al. 2004). Furthermore, Smad3 knockout tumors show reduced cell proliferation and inflammation, but increased apoptosis in response to TPA treatment (Li et al. 2004a). The observed phenomena may be attributed to Smad3 deletion-mediated blocking of TGF-β signaling, evidenced by the reduction of TGF-β-induced activator protein-1 family members and TGFα in TPA treated Smad3 null cells and tissues. Moreover, tumor tissues exhibited reduced leukocyte infiltration, particularly a reduction of tumor-associated macrophage infiltration. Consistently, the pro-inflammatory cytokine, IL-1β, and the monocyte/macrophage-attracting chemokine, MCP-1, are significantly reduced in Smad3 null tumors compared to wild-type tumors (Li et al. 2004a). Therefore, TGF-β mediated inflammation appears to require Smad3, which could explain the difference between Smad3-mediated tumor suppressive effects inside the keratinocytes and Smad3-mediated oncogenic effects on the tumor stroma.

9.2.4.3 The Role of Smad4 in Skin Carcinogenesis

To avoid embryonic lethality caused by germline deletion of Smad4 (Sirard et al. 1998; Yang et al. 1998), mouse models with Smad4 deletion specifically targeted at keratinocytes have been established (Bornstein et al. 2009; Li et al. 2003;

Qiao et al. 2006; Yang et al. 2005). Smad4 deficient skin develops progressive alopecia with hair follicle degeneration without affecting keratinocyte differentiation (Owens et al. 2008; Yang et al. 2005). Molecular mechanisms for this pathological alteration are attributed to the loss of Smad4 that blocked Smad1/5 mediated desmoglein-4 transcription (Owens et al. 2008). When Smad4 was deleted in epidermal keratinocytes, mammary epithelium or head and neck epithelium, all mice developed spontaneous SCC (Bornstein et al. 2009; Li et al. 2003; Yang et al. 2005). These findings further confirmed that Smad4 mutation acts as an oncogenic factor (Hahn et al. 1996; Thiagalingam et al. 1996). Tumors in Smad4 knockout mice have reductions in phosphatase and tensin homolog (PTEN) and $p21^{CIP/WAF}$, but upregulation of AKT, increased cell proliferation and nuclear accumulation of cyclin D1 (Qiao et al. 2006). In the established Smad4/PTEN double knockout mice, skin tumor formation was significantly accelerated compared to Smad4 deletion alone (Qiao et al. 2006; Yang et al. 2005). When Smad4 and PTEN genes were simultaneously deleted in epithelial cells of the upper digestive tract, forestomach tumor formation was also accelerated (Teng et al. 2006). Thus, Smad4 and PTEN act synergistically to regulate epidermal proliferation and differentiation. Loss of both genes may contribute to the initiation and promotion of tumorigenesis (Teng et al. 2006; Yang et al. 2005). The tumor suppressive role of Smad4 is also shown by the deletion of Smad4 in oral epithelia, which resulted in spontaneous head and neck SCC with downregulation of Fanc/Brca pathway genes, which increased genomic instability and inflammation (Bornstein et al. 2009). Thus, there are several critical differences between Smad2 and Smad4 deletion in the epithelium. Smad2 deletion in the epidermis promoted EMT at quite an early stage and caused significant increase in SPCC formation in chemical carcinogen-initiated skin cancer, but Smad2 loss itself is insufficient to initiate tumorigenesis. Increased angiogenesis observed in chemically induced Smad2−/− tumors was caused by directly upregulating HGF through recruiting Smad4 onto the SBE site of the HGF promoter (Hoot et al. 2010). Smad4 deletion in epithelial cells could initiate spontaneous carcinoma development by downregulation of PTEN or Fanc/Brca genes. EMT phenomena or SPCC was seldom observed in the Smad4 deletion tumors. Furthermore, Smad4 deleted but not Smad2 deleted tumors have increased expression of TGF-β1 and VEGF, which contributes to increased angiogenesis and inflammation (Bornstein et al. 2009; Owens et al. 2010; Qiao et al. 2006; Yang et al. 1998).

9.3 TGF-β and Systemic Sclerosis

SSc, also known as scleroderma, is a chronic and fatal autoimmune disease characterized by excessive extracellular matrix accumulation in connective tissues. SSc affects the skin and internal organs, including the lungs, gastrointestinal tract, and heart. Clinically, SSc is classified into two major types: diffuse SSc, characterized as cutaneous scleroderma with at least one internal organ involvement, and limited cutaneous scleroderma, characterized as limited skin scleroderma without internal organ involvement. The major pathologic feature of SSc is excess collagen deposition in the extracellular matrix. Although the mechanism of skin fibrosis remains

unknown, abnormal dermal fibroblast activation and chronic inflammation have been implicated in the pathogenesis of SSc. TGF-β has been shown to promote fibroblast proliferation (Wynn 2007), stimulate the production of collagen by fibroblasts (Bettinger et al. 1996; Wu et al. 2012), and induce inflammation in the skin (Li et al. 2004b). Therefore, accumulated evidence indicates that TGF-β signaling plays a key pathogenic role in SSc.

9.3.1 Increased TGF-β Expression in SSc Lesions

Histologically, fibrosis is a cardinal feature of SSc, but vasculopathy and inflammatory infiltration may also be observed in the early stages of SSc (Beyer et al. 2012; Gabrielli et al. 2009; Varga and Abraham 2007). Fibrosis is characterized by excessive collagen formation in connective tissue, produced by activated fibroblasts. Since TGF-β is shown to promote fibroblast proliferation and enhance collagen production in normal human fibroblasts, TGF-β has been implicated in the pathogenesis of fibrotic disorders (Varga and Jimenez 1986). SSc patients have high levels of plasma TGF-β, and fibroblasts derived from SSc patients display elevated levels of TGF-β mRNA expression (Higley et al. 1994; Vuorio et al. 1991). Abnormal expression of TGF-β is shown to be involved in SSc skin lesion formation (Gay et al. 1992; Gruschwitz et al. 1990; Rudnicka et al. 1994). Increased TGF-β1 was also found in endothelial cells at the early stages of SSc skin (Gabrielli et al. 1993). For the three isoforms of TGF-β (1, 2, and 3) in mammals, the mRNA for each has been detected in inflamed areas of either localized or diffused SSc, but not in sclerotic or healthy skin. However, TGF-β1 and TGF-β2 proteins were confirmed in the inflammatory skin of patients, whereas TGF-β3 protein appears to be present in the dermis of both patients and healthy controls, indicating a reduced specificity of TGF-β3 in the pathogenesis of SSc (Querfeld et al. 1999). Furthermore, TGF-β2 mRNA was found to be co-localized with pro-α1(I) collagen expression around dermal blood vessels in the inflammatory stage of SSc, but no expression of TGF-β2 or pro-α1(I) collagen was found in the dermis of fibrotic stage patients, suggesting that TGF-β2 might be more important in the pathogenesis of early SSc (Kulozik et al. 1990). In several established SSc animal models, increased TGF-β acts as a key mediator in fibrosis formation, and the reduction of TGF-β expression by several therapeutic strategies shows antifibrotic effects (Artlett 2010; Batteux et al. 2011; Iwamoto et al. 2011; Rosenbloom et al. 2010; Yamamoto 2010). These experiments further verified the role of TGF-β in the pathogenesis of SSc.

9.3.2 Upregulation of TGF-β Receptors in SSc Fibroblasts

It was also reported that alteration of TGF-β receptors occurred in SSc fibroblasts. Increased TGF-βRI and TGF-βRII mRNAs were found in cultured fibroblasts derived from SSc patients compared to healthy control fibroblasts. Consistently,

overexpression of either TGF-βRI or TGF-βRII significantly increases α2 (I) collagen promoter activity in transient transfection assays in dermal fibroblasts (Kawakami et al. 1998). Furthermore, both TGF-βRI and TGF-βRII increased in dermal fibroblasts and inflammatory cells around vascular blood in localized scleroderma and SSc lesional skin in comparison with healthy controls (Kubo et al. 2001, 2002). These studies suggest that overexpression of TGF-βRI and TGF-βRII in dermal fibroblasts results in autocrine TGF-β signaling, which plays a pathological role in SSc development. However, these findings have not been fully verified by other studies. One study reported that SSc derived fibroblasts have high levels of TGF-β RI expression, but 30 % decreased TGF-βRII expression compared to normal healthy skin derived fibroblasts (Pannu et al. 2004). Furthermore, normal fibroblasts infected with adenoviral-TGF-βRI but not adenoviral-TGF-βRII demonstrated increased basal level of type-I collagen expression. They concluded that increasing the ratio of TGF-βRI to TGF-βRII contributed to collagen formation involved in the pathogenesis of SSc (Pannu et al. 2004). Perturbation of TGF-βRI and TGF-βRII in the pathogenesis of fibrosis has also been seen in animal models of SSc. In the induced TGF-βRI (βRI^{CA}/Cre-ER) mouse models, postnatal induction of TGF-βRI in fibroblasts resulted in multiple organ fibrosis mimicking features of SSc in humans (Sonnylal et al. 2007). When constitutively active TGF-βRI was induced in fibroblasts in βRI^{CA}/Cre-ER transgenic mice, the mice developed generalized dermal fibrosis in their skin and a small-vessel vasculopathy with both smooth muscle and endothelial abnormalities in the lungs and kidneys (Sonnylal et al. 2007). Additionally, overexpression of dominant-negative TGF-βRII in fibroblasts in TGF-βRIIΔk-fib mice also caused lung and skin fibrosis as endogenous TGF-βRI was activated in transgenic fibroblasts (Denton et al. 2003, 2005; Hoyles et al. 2008).

9.3.3 Abnormal Expression of Smads in SSc

Type I collagen proteins, which consist of glycine- and proline-rich two α1(I) chains (*COL1A1*) and one α2(I) chain (*COL1A2*), have been recognized as major excess protein in SSc lesion (Jinnin 2010). *COL1A2* genes contain Smad binding sites at their promoters. Transient overexpression of Smad3 and Smad4, but not Smad1 or Smad2, causes trans-activation of the human *COL1A2* promoter in normal human dermal fibroblasts (Chen et al. 1999; Dennler et al. 1998; Zhang et al. 2000). In vitro studies demonstrated that scleroderma-derived fibroblasts had increased *COL1A2* promoter activity with high levels of Smad3 binding to the promoter compared to normal control fibroblasts (Jinnin et al. 2006). Compared to fibroblasts derived from healthy adult volunteers, increased Smad3 expression is found in fibroblasts from scleroderma patients (Mori et al. 2003). Since subcutaneous injection of bleomycin into mouse skin for 4 weeks results in fibrosis in the skin and lung, bleomycin-induced fibrosis has been extensively used to study the pathogenesis of SSc (Yamamoto 2010). When the bleomycin-inducing sclerosis protocol was applied to Smad3 knockout mice, inflammatory infiltration and TGF-β expression in the dermis at an early time point were similar in Smad3 knockout and wild-type mice.

However, by day 28 after injection, lesional skin from Smad3 knockout mice exhibited attenuated fibrosis, lower synthesis and accumulation of collagen, and reduced collagen gene transcription in situ, compared to wild-type mice (Lakos et al. 2004). These data support the idea that Smad3 mediates TGF-β-induced fibrosis in SSc thus targeting Smad3 signaling could at least in part ameliorate scleroderma. Smad7, as an antagonist of the TGF-β signaling pathway, has been reported to inhibit α1 (I) collagen production in normal human fibroblasts and decrease α-smooth muscle actin expression in hypertrophic scar-derived fibroblasts (Kopp et al. 2005). Reduced Smad7 functions, due to either reduced expression or functional defect, have been observed in SSc. In one study, SSc lesions or SSc derived fibroblasts have reduced Smad7 expression but increased Smad3 expression in comparison with that of normal skin or fibroblasts, indicating that Smad7 deficiency and Smad3 upregulation attribute to upregulated TGF-β signaling in scleroderma (Dong et al. 2002). In a separate study, SSc lesions and SSc derived fibroblasts were found to express high levels of Smad7 and TGF-βRI, and TGF-β signaling Smads remained unchanged compared to normal controls (Asano et al. 2004). Further analysis showed that the inhibitory effect of Smad7 on human α2(I) collagen promoter activity was completely impaired in SSc fibroblasts, suggesting that increased Smad7 in SSc fibroblasts may be caused by autocrine TGF-β signaling (Asano et al. 2004). The discrepancy of Smad7 expression levels in SSc skin and fibroblasts has also been reported by other studies (Kreuter et al. 2006; Zhu et al. 2012) and may be due to different sample sources or different stages of SSc (Asano et al. 2004). Recent reports indicate that the Smad1 pathway may also be involved in the pathogenesis of SSc. The first evidence came from a study on forced TGF-βRI expression-induced fibrosis, which upregulates collagen production and CCN2 (CTGF) via the TGF-βRI/Smad1 and ERK1/2 pathways (Pannu et al. 2007). The same group later showed that total and phosphorylated Smad1 levels were significantly elevated in SSc skin biopsy samples and cultured SSc fibroblasts and correlated with elevated CCN2 protein levels. Furthermore, this group identified that Smad1 was a direct activator of the CCN2 gene through binding the GC-motif in the CCN2 promoter. SSc fibroblasts exhibited elevated CCN2 promoter activity, which is correlated with the level of increased Smad1. Knocking down Smad1 in SSc fibroblasts reduced production of CCN2 and collagen (Pannu et al. 2008). Therefore, it is likely that Smad1 and Smad3 play complementary roles in the pathogenesis of SSc.

9.3.4 TGF-β Regulates Other Fibrotic Factors Implicated in SSc

In addition to TGF-β, many cytokines, chemokines, and growth factors have been implicated in the pathogenesis of SSc. However, TGF-β seems to still be a master regulator in SSc as evidenced by its crosstalk with other molecules during SSc fibrosis. The platelet derived growth factor (PDGF) pathway has long been

recognized as another key player in fibrotic diseases. PDGF regulates many aspects of fibrotic diseases including promoting myofibroblast proliferation and stimulating production of inflammatory cytokines and ECM. In SSc, TGF-β enhances the function of PDGF by upregulating expression of PDGF receptors in fibroblasts (Bonner 2004). c-Abelson (c-Abl), a non-receptor tyrosine kinase associated with chronic myelogenous leukemia (CML), is produced by TGF-β-activated fibroblasts and is also involved in TGF-β-induced fibrotic processes (Bhattacharyya et al. 2009; Rosenbloom et al. 2010). Inhibition of c-Abl by dasatinib or nilotinib has shown antifibrotic effects (Beyer et al. 2012). CCN2 (CTGF), early growth response gene I (EGR1), and Endothelin 1 (ET-1) are all induced by TGF-β and upregulated in SSc skin. All have been shown to mediate TGF-β-induced fibrosis. Targeting CCN2, EGR1 (e.g., imatinib) or ET-1 (e.g., Bosentan) may become new therapeutic strategies for treating fibrotic diseases (Bhattacharyya et al. 2012).

9.4 TGF-β and Cutaneous Hypertrophic and Keloid Scarring

Hypertrophic scarring and keloid scarring are frequently observed in clinical practice. Hypertrophic scarring occurs when the raised scar is limited to within the margin of the original injury, while keloid scarring is defined as a continually growing scar extending beyond the original injury. Scarring is usually the outcome of normal wound healing and can regress spontaneously after injury; however, hypertrophic scars caused by acute injuries such as severe burns usually result in life-long physical disability. Keloid scarring has been shown to have a strong genetic predisposition and rarely regresses (Tuan and Nichter 1998). Clinically, hypertrophic scarring and keloid scarring cause the patient physical and psychological distress and can significantly affect the patient's quality of life (Shih and Bayat 2010). Causally, both result from overgrowth of fibrous tissue characterized by excess production of ECM and collagen formation after skin damage due to injury or surgery (Seifert and Mrowietz 2009; Tziotzios et al. 2012). Many cytokines and growth factors are proposed to be involved in scar formation; TGF-β and its signaling pathway, which influences all aspects of wound healing, play key roles in regulating scar development (Seifert and Mrowietz 2009).

9.4.1 TGF-β and Scar Free Wounds

Previous studies have demonstrated that fetal wounds heal without scar formation in humans and various experimental animal models (Bullard et al. 2003). Compared to adult wounds, fetal wounds are characterized by fine and reticular collagen deposition with less cross-linking, less inflammation, higher hyaluronic acid

concentration, and a greater ratio of collagen type III to type I (Namazi et al. 2011). Multiple differences in molecules and gene expression have been identified between fetal wounds and adult wounds (Larson et al. 2010; Namazi et al. 2011). Among them, the role of TGF-β has been extensively studied. The expression of TGF-β1 and TGF-β2 is increased in adult wounds while levels of TGF-β1 and TGF-β2 are low in fetal wounding mouse models (Nath et al. 1994; Sullivan et al. 1995; Whitby and Ferguson 1991). Lack of TGF-β1 has also been observed in fetal platelets (Olutoye et al. 1996). To verify the pathological role of TGF-β1 and TGF-β2 in scar formation, TGF-β1 and TGF-β2 neutralizing antibodies were used in adult rat wounds, which resulted in reduced monocyte and macrophage profile, neovascularization, fibronectin, collagen III and collagen I deposition in the early stages of wound healing. Only given a single antibody to either TGF-β1 or TGF-β2 did the wound show less or no relieved scarring (Shah et al. 1995). In addition, some studies indicated that scar-less wounds have significantly increased levels of TGF-β3, while TGF-β1 levels remained unchanged (Eslami et al. 2009; Hsu et al. 2001). Administration of the TGF-β3 peptide to the wounded area exhibited an antifibrotic effect (Shah et al. 1995). Those studies clearly demonstrate isoform-specific differences of TGF-β playing different roles in wound healing and cutaneous scarring. Although several other reports have shown increased TGF-β1 and TGF-β2 in fetal wounds after acute wounding, these increases clear rapidly. This transient induction of TGF-β1 and TGF-β2 in fetal wounds might not be enough to induce inflammation (Cowin et al. 2001; Martin et al. 1993; Soo et al. 2003). Moreover, recent studies indicate that fetal fibroblasts do not exhibit TGF-β1-induced collagen production when compared with their mature counterparts (Rolfe et al. 2007). Thus, the difference in response to TGF-β stimuli between fetal fibroblasts and mature fibroblasts may also contribute to scar-free fetal wounds.

9.4.2 TGF-β and Cutaneous Scarring

The mechanism of keloid formation is still poorly understood, and no effective therapy for keloids exists. As studies have shown that TGF-β has a potent role in stimulating the proliferation of fibroblasts and causing inflammation, activation of TGF-β signaling has been postulated to play a central role in the pathogenesis of keloids. Compared to normal skin, keloid-derived fibroblasts have high expression of TGF-β1 and TGF-β2 proteins, but expression of TGF-β3 remains unchanged (Lee et al. 1999). Both TGF-β1 and VEGF expressions were increased in keloid fibroblasts and the upregulation of VEGF expression was dependent on TGF-β (Fujiwara et al. 2005). Furthermore, upregulation of TGF-β1, TGF-βRI, and TGF-βRII has been shown in burn-induced hypertrophic scar tissue in vivo (Ghahary et al. 1993; Schmid et al. 1998; Zhang et al. 1995). In addition, high levels of TGF-β signaling Smads including Smad2, 3, 4, TGF-βRI and TGF-βRII proteins were reported in keloid and hypertrophic scar tissue, and keloid-derived fibroblasts (Phan et al. 2005;

Tsujita-Kyutoku et al. 2005). In contrast, inhibitory Smads including Smad6 and Smad7 have been shown to decrease in keloid tissue (Yu et al. 2006). In vitro studies showed that TGF-β1 increases collagen production by keloid fibroblasts and promotes proliferation and migration of keloid fibroblasts (Bettinger et al. 1996; Wu et al. 2012). These results indicated the pathogenic role of TGF-β signaling in cutaneous scar formation (Mrowietz and Seifert 2009; Seifert and Mrowietz 2009). Ideally, accelerated wound healing without scars or with minimal scarring is the aim for perfect regeneration in adult skin injuries. TGF-β is produced by many cellular components involved in wound healing, including keratinocytes, platelets, and macrophages, and therefore production of TGF-β increases after injury (Singer and Clark 1999). However, the controversy surrounding the role of TGF-β in wound healing has existed for a long time and is still not resolved. Analysis identified that TGF-β3 in serum plays a crucial role in mediating keratinocyte migration and inhibiting dermal fibroblast migration, implying TGF-β3 is key factor initiating re-epithelialization at early stages of wound healing as keratinocytes are exposed to serum due to skin damage (Bandyopadhyay et al. 2006; Henry et al. 2003). In contrast, transgenic mice overexpressing the TGF-β1 transgene in keratinocytes exhibited delayed healing after burn injury or excisional wounding due to delayed re-epithelialization and significant skin inflammation (Chan et al. 2002; Wang et al. 2006; Yang et al. 2001). Consistently, TGF-β1 knockout mice showed accelerated re-epithelialization during incisional wound repair in comparison with wild-type mice (Koch et al. 2000), and transgenic mice overexpressing a dominant-negative TGF-βRII receptor or keratinocyte-specific deletion of TGF-βRII receptor also exhibited accelerated re-epithelialization in skin wounds (Amendt et al. 2002; Guasch et al. 2007). Smad3 knockout mice, in which TGF-β signaling is partially abolished, exhibited accelerated wound healing, featuring increased keratinocyte proliferation and migration, and reduced monocyte infiltration (Ashcroft et al. 1999). Overexpression of Smad2 in basal epidermal keratinocytes delayed wound healing due to a defect in basal keratinocyte migration (Hosokawa et al. 2005). These studies indicate that both ectopically and endogenously expressed TGF-β1 inhibits re-epithelialization and activation of TGF-β signaling in skin wounds might promote scar formation. Thus, a better wound healing outcome may be achieved by selectively blocking the negative effects of TGF-β1 (Massague 1999). Recently, we reported that transgenic mice undergoing temporal induction of Smad7, an antagonist of the TGF-β signaling pathway, in the epidermis during wound healing exhibited accelerated wound healing characterized by increased re-epithelialization and reduced inflammation, angiogenesis and the production of type I collagen in wound stroma. The mechanism involved in blocking TGF-β-induced epithelial proliferation arrest and inhibiting NFκB signaling in the epidermis by Smad7 (Han et al. 2011; Hong et al. 2007). These findings highlight the importance of future studies to evaluate whether temporal Smad7 application in skin wounds can be used as a therapeutic strategy for impaired wound healing related to defects in keratinocyte migration, excessive inflammation, and scarring.

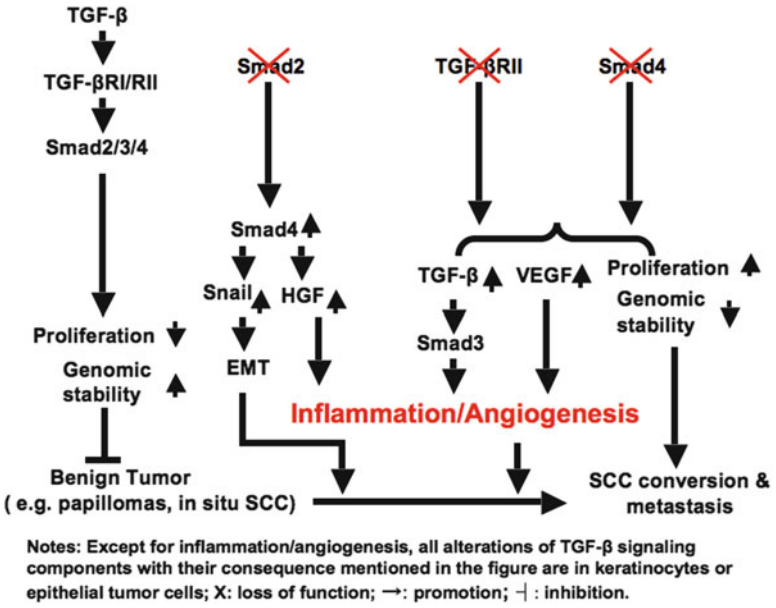

Fig. 9.1 Schematic summarizing the role of TGF-β signaling in skin carcinogenesis. TGF-β expression in skin suppresses tumor formation associated with inhibiting proliferation and maintaining genomic stability of keratinocytes. During tumor development, TGF-β-induced inflammation and angiogenesis accelerate malignant SCC conversion and metastasis. Smad2 loss in epithelia causes increased Snail and HGF by recruiting Smad4 to the promoter of Snail and HGF in tumor cells. These molecular changes lead to EMT and increasing angiogenesis, which promote SCC formation. TGF-βRII or Smad4-deficient cells express high level of TGF-β but cell itself loses the response to TGF-β-induced tumor suppression, and additional TGF-β increase stromal angiogenesis and inflammation, all of which accelerate SCC development and metastasis. Smad3 loss in skin abrogates TGF-β-induced inflammation during skin carcinogenesis

9.5 Concluding Remarks

Skin cancer and fibrotic disease are common skin diseases lacking efficient therapies. TGF-β has been implicated in the pathogenesis of both due to its differential regulation of epidermal keratinocytes, fibroblasts, and other dermal cell components. The role of TGF-β signaling in skin carcinogenesis is schematically summarized in Fig. 9.1. TGF-β first acts as a tumor suppressor in carcinogenesis as TGF-β inhibits normal keratinocyte proliferation and maintains genomic stability. However, TGF-β has also been demonstrated to play a crucial role in promoting malignant skin cancer conversion and cancer metastasis. Dominant-negative TGF-βRII transgenic mice and Smad4 deletion tumors have also increased TGF-β expression associated with tumor development and metastasis. Although SCC tumor cells express high levels of TGF-β, cancer cells lost their response to TGF-β-induced growth inhibition due to the deficient component of TGF-β signaling in tumor cells.

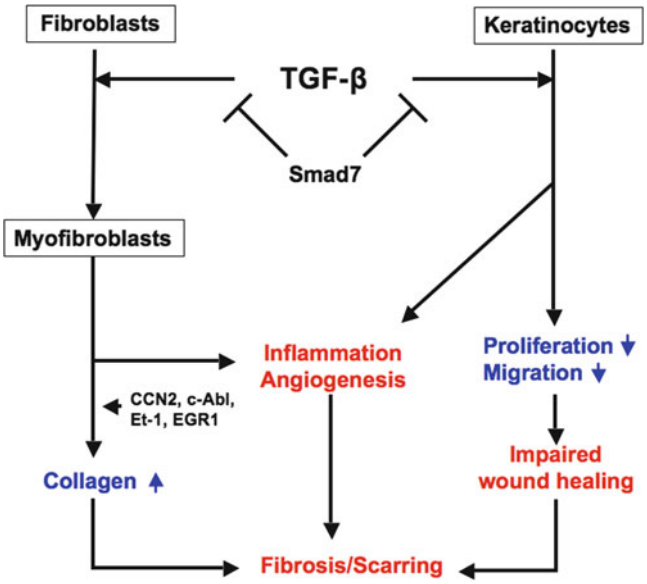

Fig. 9.2 Schematic summarizing the role of TGF-β signaling in fibrotic diseases. TGF-β acts a key player in mediating fibroblast activation to differentiate into myofibroblasts and stimulates the production of collagen via Smad3 and Smad1/Erk pathways. TGF-β also synergizes with other fibrotic factors such as CCN2, c-Ab1, Et-1, EGR1 in promoting collagen production. Excess collagen production and TGF-β-induced inflammation and angiogenesis contribute to skin fibrosis. In response to skin damage, TGF-β directly stimulates inflammation and angiogenesis in the stroma and inhibits keratinocyte migration via Smad2/3 signaling, which result in delayed wound healing and skin scarring. Overexpression of Smad7 or deletion of Smad3 reduces skin fibrosis/skin scarring and promotes wound healing through anti-inflammation, accelerating keratinocyte migration, and reducing collagen production

However, TGF-β-induced angiogenesis and inflammation in tumor stroma further accelerate tumor development and metastasis. Therefore, TGF-β contributes to either tumor inhibition or promotion depending on tumor stages. Figure 9.2 summarizes the role of TGF-β signaling in fibrotic diseases and cutaneous wound healing. In skin fibrotic diseases, it is well established that TGF-β plays a crucial role in fibrosis formation due to its ability to activate fibroblasts and stimulate collagen production in the extracellular matrix. Activation of TGF-β receptors, especially TGF-βRI, and abnormal expression of Smads have been documented in skin fibrosis. Based on wound healing studies in the fetus, the isoforms of TGF-β subtypes may have different roles in cutaneous scar formation. TGF-β1 is a negative factor in cutaneous wound healing and promotes skin scarring due to its action in fibroblast activation and induction of inflammation. Blocking TGF-β signaling may lead to novel strategies for treating impaired wound and skin fibrotic diseases in humans.

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Chapter 10
The Role of TGF-β in Cutaneous Melanoma Biology

Delphine Javelaud and Alain Mauviel

Abstract Ample evidence indicates that transforming growth factor-β (TGF-β) signaling plays a major role at various stages of carcinogenesis. While it may represent a tumor suppressor pathway at early stages of cancer progression, essentially due to its cytostatic activity, it has also become clear that TGF-β may act as a potent tumor promoter via both autocrine and paracrine mechanisms: TGF-β enhances tumor cell migration and homing to various metastatic sites, allows tumor escape from the immune system and promotes peri-tumoral vasculogenesis. This chapter reviews the current literature on the implication of TGF-β signaling in melanoma.

Keywords Invasion • Melanoma • Metastasis • TGF-β

10.1 Introduction

Melanomas arise from the transformation of melanocytes, cells that derive from melanoblasts in the neural crest that migrate and differentiate before localizing essentially in the hair follicles and in the epidermis. They produce melanin and are responsible for the pigmentation of hair and skin. Premalignant melanocytic lesions include clinicopathologic entities such as lentigo, nevi, or dysplastic nevi (Chin et al. 2006) Melanoma is the most lethal skin cancer and its incidence increases faster than any other malignancy. Although early stage melanoma patients can be treated successfully by surgical resection of the primary tumor, the prognosis for metastatic disease is dismal with an overall 5-year mortality rate of 90 %: melanomas are highly aggressive tumors that exhibit strong metastatic potential together

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with high resistance to common anticancer treatments, including chemotherapy, interleukin-2-based immunotherapy, and a combination of chemotherapy and immunotherapy.

The key malignant transition is thought to occur from radial growth phase (RGP) to vertical growth phase (VGP) of primary melanoma lesions, which results in expansive infiltration of the dermis and its constituent structures, such as blood and lymphatic vessels, presaging distant metastasis.

Genetic alterations occur early during melanoma development and contribute to melanoma development. They include inactivation of the *INK4a/ARF* melanoma susceptibility locus, activating mutation of *NRAS*, *BRAF*, and *cKIT* oncogenes, alteration/loss of *PTEN*, *p53* mutations and *CDK4* mutations [see Table 10.1 and (Dutton-Regester and Hayward 2012; Flaherty et al. 2012; Tsao et al. 2012)].

Targeted therapeutic strategies have emerged over the recent years, accompanied with new hopes to cure patients with advanced melanoma. For example, Vemurafenib (Plexxikon/Roche) or GSK2118436 (GlaxoSmithKline) are highly selective and very potent mutated BRAF inhibitors. BRAF is the most commonly activated oncogene in melanoma, with approximately 50 % of advanced melanomas harboring such mutations. About 80–90 % of the BRAF mutations found in melanoma consist of an activating substitution of glutamate for valine at position 600 of the kinase domain within the BRAF amino-acid sequence ($BRAF^{V600E}$). In a randomized phase III trial comparing Vemurafenib to the commonly used chemotherapeutic drug, Dacarbazin, Vemurafenib treatment led to a decrease of 63 % for relative risk of death and to a significant increase in overall survival (+6 months). However, some patients presented resistance to this BRAF inhibitor and new devastating progression of the disease. Identification of drug resistance mechanisms developed by tumors and rapid preclinical and clinical testing of strategies to overcome such resistance are under investigation. Ongoing trials are combining BRAF and MEK inhibitors with the goal of delaying or overcoming resistance as a result of reactivation of the MAPK pathway [reviewed in (Flaherty et al. 2012), ASCO 2012, http://chicago2012.asco.org/Abstracts.aspx]. Another drug, Ipilimumab, a blocking antibody targeting CTLA4 (Cytotoxic T lymphocyte-associated antigen4), a protein involved in the differentiation of immunosuppressive regulatory T cells, has been tested in stage IV melanoma patients. Initial results indicate a clear increase in overall survival (ASCO 2012, http://chicago2012.asco.org/Abstracts.aspx).

Given the heterogeneity of genetic alterations found in melanoma patients and the weak success of actual therapeutic treatments coupled with exacerbated resistance mechanisms, further efforts have to be realized in developing targeted therapies. In this context, numerous preclinical studies have provided convincing evidence that TGF-β and its cognate signaling cascade play a major role in the progression of malignant melanoma to metastasis and could therefore represent a relevant target for preventing melanoma development. This chapter summarizes the literature on the pleiotropic activities of TGF-β in cutaneous melanoma biology (Fig. 10.1).

Table 10.1 Selected genetic alterations in malignant melanoma thought to be involved in melanomagenesis

Gene	Functions/pathways affected	Alteration in melanoma (frequency)
Signaling factors		
BRAF	Oncogene/MAPK signaling	Point mutation (50 %)
NRAS	Oncogene/MAPK signaling	Point mutation (20 %)
MEK	Oncogene/MAPK signaling	Point mutation (1–2 %)
KIT	Oncogene/MAPK and PI3K signaling	Point mutation (<1 %)
ERBB4	Oncogene/PI3K signaling	Point mutation (15–20 %)
PTK2B	Oncogene/MAPK signaling	Point mutation (2.5 %)
NEDD9	Melanoma metastasis gene	Amplification (55–60 %)
AKT1, AKT2, AKT3	Oncogene/PI3K signaling	Point mutation (<1 %) or amplification (25 % for AKT3)
PTEN	Tumor supressor/PI3K signaling repression	Point mutation (50–60 %)/ hemizygous deletion (50–60 %) homozygous deletion (10 %)
CTNNB1	Cell adhesion/transcriptional co-activator	Point mutation (30 %)
EGFR	Oncogene/β-catenin and MAPK signaling	Amplification associated with polysomy 7 (80 %)
MET	Oncogene/promoter of cellular invasion	Amplification (47 %)
Cell cycle and apoptosis regulators		
CDK4	Promoter of cell proliferation	Point mutation or amplification (5 %)
CDKN2A/INK4A	Tumor supressor-cell cycle inhibitor	Point mutation or deletion (30 %)
CCND1	Promoter of cell proliferation	Amplification (10 %)
TP53	Tumor supressor- cyle inhibitor/ apoptosis inducer	Point mutation (5 %)
APAF1	Apoptosis inducer	Silenced/promoter inactivation (40 %)
Transcription factors		
MITF	Melanocyte-specific lineage transcription factor	Amplification (20 %)
ETV1	Oncogene/MAPK signaling and MITF induction	Amplification (10–20 %)
MYC	Oncogene/Promoter of cell proliferation	Amplification (20 %)

From Flaherty et al. (2012), Meyle and Guldberg (2009), Miller and Mihm (2006)

10.2 TGF-β Signaling Molecules in Melanoma

Increased expression and secretion of the different TGF-β isoforms in melanoma cell lines when compared with normal melanocytes has been reported by several studies (Albino et al. 1991; Krasagakis et al. 1994; Rodeck et al. 1991; 1994).

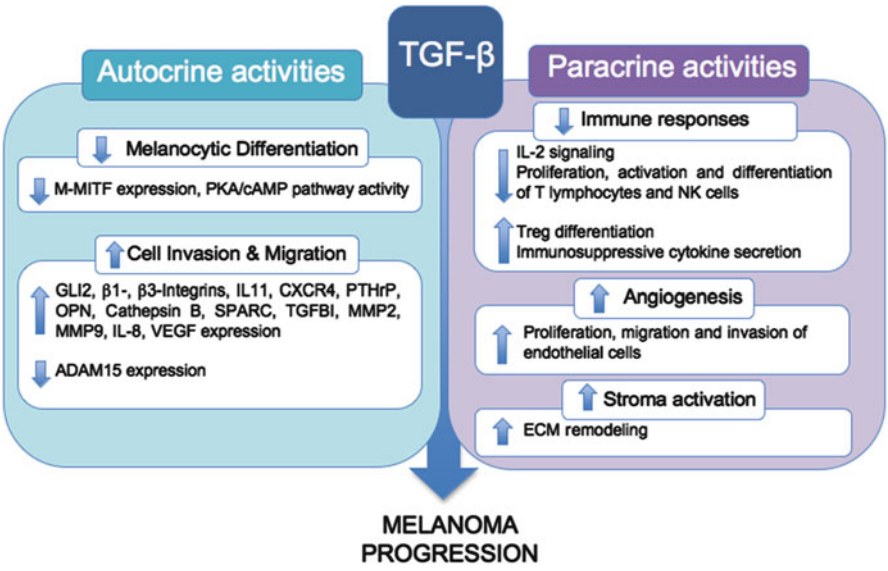

Fig. 10.1 Multiple roles of TGF-β in promoting melanoma development

In situ, TGF-β1 is secreted by normal melanocytes and melanomas at various stages, while TGF-β2 and TGF-β3 are not expressed in normal melanocytes but only heterogeneously in nevi and melanoma. Expression of TGF-β2 and TGF-β3 seems to appear early in melanoma progression and to increase with tumor progression (Van Belle et al. 1996). A correlation between TGF-β2 expression levels and tumor thickness has also been reported (Reed et al. 1994). Similarly, increased TGF-β1 and TGF-β2 plasma levels are observed at later stages of tumor development, while no significant differences have been reported between those of healthy patients and those from patients with primary or locally invasive melanoma (Krasagakis et al. 1998). Thus despite some discrepancies, all these studies point toward an increase in TGF-β expression levels that correlates with tumor progression.

Interestingly, TGF-β treatment of melanoma cells induces expression of TGF-β1 and TGF-β2 in a positive amplification loop. Moreover, patients with MMP-1 positive metastases had significantly shorter disease-free survival compared to patients with MMP-1 negative metastases (median 11.2 vs. 17.0 months, $p = 0.0383$) (Nikkola et al. 2002) and MMP-1 secreted by melanoma cell was shown to be involved in the activation of TGF-β from its latent form (Iida and McCarthy 2007).

Expression of TGF-βII receptor (TβRII) mRNA is much more heterogeneously distributed in primary melanomas compared with benign melanocytic nevi. Melanoma progression appeared not to be associated with a complete loss of *TβRII* gene expression, since all skin metastases revealed clearly detectable although heterogeneous levels of TβRII mRNA expression (Schmid et al. 1995). Melastatin (TRPM1), a member of the transient receptor potential (TRPM) cation channel

family, is robustly expressed in benign and dysplastic nevi and in melanomas in situ, but it is only variably expressed in invasive melanomas. Melastatin expression levels show widespread downregulation in melanoma metastases and are inversely correlated with metastatic potential and good prognosis in melanoma (Duncan et al. 1998; 2001). Intron 6 of the *TRPM1* gene hosts the gene for miR-211, a microRNA whose expression is restricted to the melanocyte lineage (Gaur et al. 2007). miR-211 and melastatin share the same promoter and are expressed coordinately in melanocytes and melanomas. It has been described that TβRII is one of the direct targets of miR-211 (Levy et al. 2010), suggesting that epigenetic events may control the expression and activity of the TGF-β in melanoma cells during disease progression.

CD105/endoglin, a co-receptor associated with TβRII that modulates the activity of the latter, is heterogeneously expressed in small clusters of tumor cells within primary melanomas and in intradermal nevi and metastatic melanoma lesions (Altomonte et al. 1996; Pardali et al. 2011).

Several studies have described a functional Smad signaling in melanoma. Basal phosphorylation of Smad3 is detectable in vitro by western blot analysis of whole melanoma cell extracts, demonstrating a constitutive activation of the TGF-β receptors. Furthermore, a pan-TGF-β antibody was shown to inhibit basal Smad3/Smad4-dependent transcriptional activity in melanoma cells (Javelaud et al. 2007; Rodeck et al. 1999). Also, exogenous TGF-β was shown to induce further a Smad3/Smad4 transcriptional response, demonstrating the functionality of the entire signaling cascade (Javelaud et al. 2007; 2008; Rodeck et al. 1999). Frequent nuclear phosphorylation of Smad2 has been detected in clinical specimens of benign and malignant cutaneous melanocytic neoplasms (Lo and Witte 2008), suggesting that the TGF-β pathway is constitutively activated in melanomas. Yet, another study showed that Smad2 phosphorylation correlated with low tumor thickness but not with overall survival and development of metastases suggesting that it would not be a useful prognostic marker (Mnich et al. 2007). This indicates the need for further studies to characterize both the phosphorylation and cellular localization of Smad2, Smad3, and Smad4 in clinical samples to determine their exact activation state throughout the various stages of melanoma progression. Interestingly, it has also been shown that phosphorylation of Smad2 in melanoma cells is associated with Smad2 phosphorylation in neighboring keratinocytes, indicating that melanoma-derived TGF-β affects the microenvironment and adjacent cells in a paracrine fashion (Mnich et al. 2007). Genome-wide expression analysis of nearly a hundred human melanoma cell lines has identified two groups with very distinct gene expression profiles: the first one is characterized by high expression of neural crest and melanocytic differentiation markers, the other one is characterized by the expression of a number of genes associated with a more aggressive phenotype, whose concomitant expression is reminiscent of a TGF-β signature (Hoek et al. 2006). These two groups present very distinct behaviors: the melanocytic differentiation signature is associated with strong proliferation but weak invasive capacity, while the TGF-β signature characterizes highly invasive melanoma cell lines with low proliferation rate (Hoek et al. 2006).

10.3 Resistance to TGF-β Induced Cell Cycle Arrest in Melanoma Cells: A Complex, Somewhat Unresolved Issue

TGF-β exerts potent anti-proliferative activity on normal melanocytes but has weak or no effect on melanoma cells. During tumor progression, melanoma cells acquire a growing resistance to TGF-β-dependent growth inhibition (Krasagakis et al. 1999). The latter may be explained by attenuation or inhibition of the TGF-β pathway in these cancer cells. For example, CD105 expression is correlated with cell sensitivity to TGF-β treatment, suggesting that expression of this co-receptor may modulate the cellular response to TGF-β (Altomonte et al. 1996). Recently, Yilmaz et al. described that TGF-β induction of DLX-2 (Distal-less homeobox) attenuates growth suppressive TGF-β signaling in a negative feedback loop (Yilmaz et al. 2011). Expression of filamin, a cytoskeletal actin–binding protein, has also been suggested to repress TGF-β signaling by direct interaction with Smad2 and Smad4 (Sasaki et al. 2001). SKI and SNON oncoproteins are direct repressors of Smad2/3 transcriptional activity and are highly expressed in melanoma cells lines as well in melanoma samples (Javelaud et al. 2011; Reed et al. 2001). In some melanoma cell lines, downregulation of SKI expression using antisense SKI vectors or shRNA restored TGF-β-mediated growth inhibition through $p21^{CIP1}$ induction and inhibited subcutaneous tumor growth in xenograft experiments (Chen et al. 2009; Reed et al. 2001). MIA (melanoma inhibitory activity) is a secreted protein expressed in melanomas but not in melanocytes and is associated with tumor progression in vivo (Bosserhoff et al. 1997). Rothhammer et al. have shown that MIA induces SKI and SNO expression, leading to reduced Smad2/3 expression in melanoma cells (Rothhammer and Bosserhoff 2006).

Despite overexpression of different proteins known to repress TGF-β signaling, mechanisms of resistance to TGF-β-induced cell growth arrest are likely more complicated than due to a single inhibitory protein. Autocrine activation of the pathway in melanoma cell has been documented in a variety of settings (Lo and Witte 2008; Rodeck et al. 1999). High phosphorylation levels of Smad3 and Smad2 linker domain phosphorylation have been described in both melanoma cell lines and clinical melanoma specimens (Cohen-Solal et al. 2011). The latter phosphorylation event specifically inhibits the expression of the cell cycle inhibitors $p21^{CIP1}$ and p15, and resulting cell growth arrest by TGF-β, but does not alter the induction of other TGF-β target genes such as PAI-1 (Cohen-Solal et al. 2011). At this stage, the mechanisms responsible for Smad2 linker domain phosphorylation are not fully understood and several studies have implicated high levels of SKI, GSK3, CDK, and MAPK (p38, JNK and ERK) in the phosphorylation of Smad2/3 linker domains (Cohen-Solal et al. 2011; Lin et al. 2010). In particular, melanomas are characterized by hyperactivity of the MEK/ERK cascade, often induced by activating mutations of the BRAF and NRAS oncogenes (Chin et al. 2006; Flaherty et al. 2012; Tsao et al. 2012). ERK activation may contribute to the phosphorylation of Smad2/3 linker domains and elicit TGF-β-induced growth arrest resistance.

Shellman et al. have shown that forced overexpression of NRAS in primary melanoma cell lines counteracts TGF-β-induced growth inhibition by preventing the accumulation of hypophosphorylated Rb protein (Shellman et al. 2000).

Another important protein that may be implicated in the loss of growth inhibition by TGF-β is Id2, a positive regulator of cell growth and plays a critical role prompting G1/S cell cycle progression. Id2 inhibits CDK inhibitors expression by interfering with bHLH, ETS and PAX transcription factor activity and growth suppressing activity of pRb by direct interaction. Id2 is negatively regulated by TGF-β, yet Schlegel et al. have shown a differential inhibition of Id2 by TGF-β in melanoma cells, depending on whether they are of the proliferative or invasive type (Schlegel et al. 2009).

During melanoma initiation and progression, a number of genetic and epigenetic alterations occur, including amplification of c-MYC or cyclin D1 overexpression, hyperactivation of CDK4 due to loss of the CDK inhibitor p16 function or activating mutations in the CDK4 gene, alterations in the activity of CDK inhibitors such as $p21^{CIP1}$, p27, or p15. All of them may participate in a cellular activation state whose endpoint is an attenuation of the TGF-β anti-proliferative effects.

PAX3, a paired-box transcription factor involved in melanocyte differentiation and survival, could also participate in resistance to TGF-β-induced cell growth arrest. Its forced overexpression in melanoma cells attenuates TGF-β anti-proliferative activity and TGF-β represses PAX3 expression (Yang et al. 2008).

10.4 Promotion of Melanoma Aggressiveness by Autocrine TGF-β Signaling

Melanoma cells secrete high levels of TGF-β ligands and most of them are largely insensitive to their anti-proliferative activity. Autocrine TGF-β is also involved in a number of additional cellular processes, as it can act on tumor cells and their microenvironment by autocrine and paracrine mechanisms that contribute to tumor development and aggressiveness.

Cell autonomous effects of TGF-β in epithelial tumor cells have been long identified. In particular, this pathway is involved in the promotion of epithelial to mesenchymal transition (EMT), remodeling of extracellular matrix, cell migration, and cell survival (Tian et al. 2011). EMT takes place during essential processes of embryogenesis such as gastrulation. Coordinated modifications in gene expression promote cell migration through changes in the expression of cell–cell adhesion molecules of the cadherin family. Consequently, epithelial cells acquire a fibroblast-like phenotype (Davies et al. 2002). This phenomenon is also observed during the metastatic development of epithelial tumors. Melanoma derives from the neuroectoderm, yet melanoma cells undergo gene expression changes and acquire a fibroblast-like phenotype similar to what is observed in epithelial tumor. This melanoma-specific phenomenon may be qualified a pseudo-EMT.

In melanoma, TGF-β has been shown to promote tumor invasion via induction of β1- and β3-integrins and inhibition of *CDH1*, promoting pseudo-EMT (Janji et al. 1999). There is also strong evidence for a direct role of autocrine TGF-β signaling in melanoma development and progression. For example, overexpression of the inhibitory Smad7 in melanoma cells reduces their capacity to form colonies in an anchorage-independent manner, dramatically inhibits the secretion of matrix metalloproteinases MMP-2 and MMP-9 and their capacity to invade Matrigel, and delays tumor growth in a subcutaneous injection model in *nude* mice (Javelaud et al. 2005). Furthermore, inhibition of constitutive TGF-β activity by Smad7 overexpression dramatically reduced experimental bone metastasis development in mice, by preventing TGF-β induction of genes known to mediate osteolytic breast cancer bone metastases, such as IL-11, PTHrP, CXCR4, and OPN (Javelaud et al. 2007). In a model of in vivo human skin grafting onto mice, it has been observed that overexpression of Smad7 in melanoma cells blocks invasion with melanoma cells being kept in close proximity to the dermal-epidermal junction (DiVito et al. 2010). Smad7 was found to increase N-cadherin expression in melanoma cells, thus promoting homotypic interactions between melanoma cells and dermal fibroblasts. Smad7 overexpression also inhibited β-catenin T41/S45 phosphorylation, resulting in the stabilization of β-catenin (DiVito et al. 2010). Of note, increased cytoplasmic/nuclear β-catenin expression has been linked with good prognosis and increased survival of melanoma patients (Chien et al. 2009).

Treatment of melanoma cell lines with SD-208, a highly specific small molecule inhibitor of TβRI, blocks in vitro TGF-β induction of Smad3 phosphorylation, TGF-β induced Smad3/Smad4–driven transcription, as well as invasion through Matrigel. SD-208 was also shown to reduce the development of experimental osteolytic bone metastases following intracardiac inoculation of tumor cells in *nude* mice (Mohammad et al. 2011). SD208 was also found to reduce the growth of established bone metastases in the same model. Together with the data obtained with Smad7 overexpression, these experiments demonstrate that inhibition of constitutive activation of TGF-β signaling may represent a valuable therapeutic option to prevent melanoma progression to metastases and inhibit the growth and dissemination of existing metastases.

We have identified the transcription factor GLI2 as a direct transcriptional target of TGF-β signaling in a variety of normal and transformed cell lines, including melanoma (Dennler et al. 2007; 2009). GLI2 is known to be a mediator of the Sonic Hedgehog pathway and numerous studies have established its role in cancer development (Javelaud et al. 2012). In melanoma cells, we found that GLI2 regulates the invasive capacity via downregulation of E-cadherin, a protein that plays a critical role in maintaining melanocyte interactions with epidermal keratinocytes, and upregulation of metalloproteinases, MMP2 and MMP9, that contribute to basement membrane invasion by tumor cells (Alexaki et al. 2010). GLI2 has also been shown to induce the expression of proteins known to participate in the metastatic process, such as WNT5a (Dissanayake et al. 2008; Reddy et al. 2001) and PTHrP (Sterling et al. 2006).

Numerous studies described modulation of gene expression by the TGF-β pathway, participating in melanoma development. For example, TGF-β induces expression of integrins, proteases such as Cathepsin B, and that of matrix remodeling proteins such as SPARC involved in the promotion of cellular invasion and migration, and represses the expression of ADAM15 (A Disintegrin And Metalloproteinase) in cooperation with IFN-γ (Ungerer et al. 2010). ADAM15 is expressed in melanocytes and endothelial cells of benign nevi and melanoma tissues and is downregulated in melanoma metastases compared to primary melanoma lesions (Ungerer et al. 2010). It seems to have tumor suppressor function in melanoma, as tail vein injection of recombinant disintegrin domain of ADAM15 together with mouse melanoma cells reduces the formation of lung metastases in mice (Trochon-Joseph et al. 2004). Furthermore, overexpression of ADAM15 has been shown to reduce migration and invasive capacity of melanoma cells in vitro, probably driven by induction of α5β1 integrin expression and increased adhesion to fibronectin (Chen et al. 2008; Ungerer et al. 2010).

TGFBI (transforming growth factor-β induced) is a TGF-β1-inducible ECM protein that plays a role in the invasive growth of melanoma cells (Nummela et al. 2012). It is highly expressed during melanoma VGP and in metastatic cell lines, has anti-adhesive properties and promotes the growth of subcutaneous tumor xenografts in mice. TGFBI localizes at tumor edges, together with fibrillar fibronectin/tenascin-C/periostin structures that characteristically surround melanoma cells at the invasive front. Both in tumors generated in nude mice and in human melanoma metastases.

Genome wide expression analysis of a large series of human melanoma cell lines revealed distinct groups characterized by specific invasive and proliferative behaviors. On the one end, highly proliferative cell lines that express neural crest and melanocytic differentiation markers, such as MITF, TYR, and MLANA, were found, while another group was composed of cell lines that grow slower than the former, are more invasive, and display high expression of a set of genes controlled by the TGF-β pathway, including TGFBI, SERPINE1, and CTGF, to cite a few (Hoek et al. 2006). These extreme phenotypes were indistinguishable by their BRAF or NRAS mutation status. It thus appeared from this broad study that cell autonomous TGF-β activation is associated with a characteristic TGF-β gene signature together with an invasive cell behavior. The genetic context may still contribute to TGF-β-induced invasive behavior of melanoma cells, as it has been shown that forced TGF-β pathway activation in immortalized melanocytes via overexpression of a constitutively activated TβRI only promotes invasion into the dermis of organotypic human skin cultures in the presence of mutated $BRAF^{V600E}$ and PTEN deficiency (Lo and Witte 2008). This was also associated with increased levels and activities of MMP-2 and MMP-9.

One important point to keep in mind is that human melanoma tumors are, in essence, highly heterogeneous and present both invasive and proliferative regions simultaneously. These behaviors are described as reversible and may be under the control of signals originating in the tumor microenvironment (Hoek et al. 2008).

Both primary and metastatic melanomas contain a heterogeneous mix of more or less differentiated cells. Strikingly actively disseminating cells are more uniform with low levels of pigment and high levels of TGF-β2 expression (Pinner et al. 2009). Several lines of evidences have suggested that TGF-β ligands negatively control melanocyte differentiation. Melanocytes in the epidermis rarely proliferate and produce melanin pigments that protect the skin from deleterious ultraviolet light (UV), while melanocytes in the hair follicles repeatedly proliferate and differentiate for hair pigmentation in every hair cycle. The hair follicle repeats its cyclic regeneration and regression with alternating phases, anagen (growing phase), catagen (regressing phase), and telogen (resting phase) to regrow new hair (Fuchs 2007). The fates of follicular melanocytes, including their proliferation, differentiation, and death all happen in synchronization with hair cycle progression (Foitzik et al. 2000; Nishimura et al. 2002). The catagen phase is an apoptosis-driven process accompanied by terminal differentiation, proteolysis, and matrix remodeling. TGF-β plays an important role in catagen regulation as an inhibitor of keratinocyte proliferation and as an inducer of apoptosis (Foitzik et al. 2000; Soma et al. 2003; Tumbar et al. 2004). Melanocyte stem cells reside with keratinocyte stem cells in the hair follicle bulge enriched in active TGF-β. Melanocyte stem cell quiescence is preceded by activation of TGF-β signaling, loss of Ki67 expression, downregulation of melanogenic gene expression, and dramatic morphologic changes from dendritic shape into a slender, oval shape with shrinkage resulting from an increased nuclear/cytoplasmic ratio (Nishimura et al. 2010). Upregulation of TGF-β1/2 expression in the niche area and phospho-Smad2 expression were detected prior to the morphologic changes, downregulation of melanogenic genes, and loss of Ki67 expression by melanocyte stem cells. Activation of TGF-β signaling plays dual roles in melanocyte stem cell maintenance: (a), through inhibition of stem cell differentiation in the stem cell niche and (b), via induction of stem cell quiescence that requires BCL2 expression for cell survival (Nishimura et al. 2010). TGF-β may also prevent melanocyte maturation through a direct repression of M-MITF expression (Kim et al. 2004; Pierrat et al. 2012). M-MITF is a master transcription factor of the melanocytic lineage that controls cell survival, migration, and differentiation. This factor directly activates expression of melanogenic genes such as those encoding tyrosinase, TRP1, and TRP2. TGF-β inhibits M-MITF promoter activity by two different mechanisms: repression of CREB activity through an inhibition of cAMP/PKA signaling and induction of GLI2, the latter directly binding to the MITF promoter to repress transcription by mechanisms that remain to be identified (Pierrat et al. 2012). Proteolysis of tyrosinase has also been involved in melanogenesis inhibition by TGF-β (Martinez-Esparza et al. 1997).

TGF-β also induces dedifferentiation of melanoma cells and promotes cell migration and invasion, thereby promoting metastasis. This dedifferentiation is reversible, transient, and dynamic and probably dependent upon hypoxia and the tumor microenvironment (Hoek et al. 2008; Pinner et al. 2009).

10.5 The Role of Tumor-Derived TGF-β on the Microenvironment

Paracrine effects of TGF-β on surrounding cells in the tumor microenvironment may be advantageous for melanoma cells. Two main roles are to be pointed out. Suppressive effects of TGF-β on the immune system may allow tumor cells to escape from immune surveillance, and pro-angiogenic properties of TGF-β could support nutrition of the tumor and facilitate metastasis. In addition, stimulation of stromal cells by TGF-β leads to increased production of reciprocally paracrine-acting growth factors. Also, melanoma cells can modulate their surrounding stroma for their own benefits through the paracrine activity of TGF-β. Stimulation of production of ECM proteins such as collagen, fibronectin, tenascin, and α2-integrin, by stromal fibroblasts provides a scaffold for melanoma cells to adhere and migrate, leading to increased survival and metastasis formation (Berking et al. 2001). Finally, modulation of the expression of proteases and their inhibitors by TGF-β likely facilitates invasion by means of stromal remodeling.

Aberrant TGF-β signaling in mice results in large vascular and endothelial defects, demonstrating the central role of TGF-β in embryonic vascular morphogenesis and in the establishment and maintenance of vessel wall integrity (Goumans et al. 2003). TGF-β can regulate the initiation and development of new blood vessels as well as the maturation of newly formed vessels. These processes involve not only stimulation of proliferation, migration, and invasion of endothelial cells during initiation of angiogenesis but also increase in endothelial cell adhesion, basement membrane deposition and recruitment of pericytes, and vascular smooth muscle cells during vessel maturation (Tian et al. 2011). In melanoma cells, TGF-β enhances angiogenesis by activating the expression of proangiogenic factors such as Interleukin (IL)-8 and VEGF (Liu et al. 2005).

TGF-β exerts immunoregulatory functions by inhibiting the proliferation, activation, and differentiation of lymphocytes, as well as the natural killer and dendritic cell functions, all these events leading to the suppression of the antitumor immune response (Arteaga et al. 1993; Letterio and Roberts 1998; Wrzesinski et al. 2007). Noteworthy, transplantation of murine bone marrow expressing a dominant-negative TβRII in a syngeneic mouse model of melanoma leads to the generation of mature leukocytes capable of a potent antitumor response (Shah et al. 2002).

TGF-β also exerts repressory effects on IL-2 activity, the main activator of immune response: TGF-β inhibits the phosphorylation and activation of components of the JAK/STAT cascade downstream of the IL-2 receptor and expression of IL-2 targets genes such as c-MYC and Cyclin D2 (Bright et al. 1997; Nelson et al. 2003). TGF-β is also an important differentiation factor for regulatory T cells (Foxp3+ Treg), a subset of strongly immunosuppressive T cells often found in tumors, including melanoma.

MCP-1 (monocyte chemoattractant protein-1), a potent chemokine that recruits macrophages, is expressed by malignant but not normal melanocytes (Nesbit et al. 2001). Monocytes and macrophages infiltrate melanomas and produce factors

promoting tumor development and invasion, including immunosuppressive cytokines and angiogenic factors (Dirkx et al. 2006). IL-10 is also expressed in primary and metastatic melanoma lesions (Dummer et al. 1996) and elevated levels of IL-10 have been measured in sera of patients with advanced melanoma that correlate with tumor progression (Moretti et al. 2001; Nemunaitis et al. 2001). TGF-β increases both MCP-1 and IL-10 expression in melanoma cells, by means of a crosstalk between the Smad, AKT, and MAPK/ERK pathways. Thus, TGF-β enhances MCP-1 mediated monocyte/macrophage migration and IL-10 immunosuppressive functions (Diaz-Valdes et al. 2011).

10.6 Other TGF-β Family Members in Melanoma

While the above paragraphs have focused on TGF-β *stricto sensu*, other members of the TGF-β family of growth factors are also expressed and/or contribute to melanoma development. A brief overview of the cognate literature is provided below.

It has been shown that Activin inhibits the proliferation and induces apoptosis of primary melanocytes. Expression of Activin and its receptors has been detected in melanoma cell lines that show a response to exogenous Activin. Yet those cell lines were found to be resistant to Activin's anti-proliferative activity (Stove et al. 2004).

Melanoma cells, unlike normal melanocytes, express and secrete high level of Follistatin. Follistatin is a secreted protein that binds to activin and blocks activin-induced signaling (Stove et al. 2004). It was suggested that early during melanoma progression, secretion of Follistatin may protect against the cytostatic function of activin while, on the other hand, melanoma cells at a later stage may use activin, either autocrine or paracrine, to maintain a state of dedifferentiation and create a microenvironment supportive of melanoma growth.

Expression of multiple BMPs (bone morphogenetic proteins) including BMP-2, -4, -7, is upregulated in melanomas compared to nevi (Rothhammer et al. 2005), and expression of BMP-4 and BMP-7 was found to increase during disease progression. BMP-4 was found to be implicated in the control of the migratory and invasive properties of melanoma cells, as inhibition of BMP signaling by means of overexpression of the BMP inhibitor chordin, or by specific downregulation of BMP4 expression, resulted in a strong reduction of both invasive and migratory capacities of melanoma cells (Rothhammer et al. 2005), possibly via reduced MMP-1, 2, 3, and 9 expression (Rothhammer et al. 2008). Dido1 (Death inducer-obliterator 1) was recently identified as a direct and specific BMP target gene. This protein was first described as an early apoptosis regulator protein and as a tumor suppressor gene in hematological myeloid neoplasm (Jilaveanu et al. 2009). Upon apoptosis induction, Dido-1 is translocated into the nucleus and activates pro-caspase expression (Garcia-Domingo et al. 2003). It is highly expressed in the nucleus of melanoma cell lines derived from metastases (Braig and Bosserhoff 2013). Dido1 expression induced by BMP-4 signaling regulates anchorage independent growth, apoptosis resistance as well as the migratory and invasive capacities of melanoma

cells (Braig and Bosserhoff 2013). In melanocytes, BMP-4 inhibits melanogenesis by blocking Tyrosinase expression (Yaar et al. 2006). Accordingly, overexpression of Noggin, a BMP antagonist, in the epithelium of hair follicles in mice, induces a darker coat color compared to wild-type mice (Sharov et al. 2005). Inversely, BMP-2 promotes melanogenesis by inducing the expression of Tyrosinase (Bilodeau et al. 2001). In astrocytoma cells, BMP-2 also inhibits the expression of HGF (hepatocyte growth factor) (Chattopadhyay et al. 2004), a potent mitogenic, motogenic, and morphogenic cytokine for melanoma cells (Noonan et al. 2003).

While BMP7 is overexpressed in melanoma tissues, it may exert antitumor activities. For instance, it has been described that BMP7 inhibits the proliferation of both normal and malignant melanocytes, an effect that is alleviated by concomitant overexpression of Noggin (Hsu et al. 2008). Noggin expression is also correlated with an induction of Nodal and VEGF expression in a subset of but not all melanoma cells lines. Induction of these growth-promoting factors may participate in the restoration of proliferation (Hsu et al. 2008). Another study only found a minor effect of BMP7 on melanoma proliferation while potently inhibiting of both the migratory and invasive capacities of melanoma cells (Na et al. 2009).

Nodal expression is restricted to embryonic tissues, epithelial stem cells, and cancer cells. Nodal is absent in normal skin and only rarely detected in poorly invasive radial growth phase melanomas. Yet, it is observed in up to 60 % of cases of vertical growth phase primary melanoma lesions and melanoma metastases. Its expression correlates with melanoma progression and experimentally, its depletion in melanoma cells decreases anchorage-independent growth and plasticity, concomitant with a marked abrogation of tumorigenicity (Topczewska et al. 2006; Yu et al. 2010).

Cripto-1, a Nodal co-receptor, is also expressed in melanomas and contributes to invasion and proliferation of melanoma cells (De Luca et al. 2011). In addition to mediating Nodal signaling, Cripto-1 modulates signaling of other TGF-β family ligands, including GDF-1, GDF-3, Activin, and TGF-β.

MIC-1 (macrophage inhibitory cytokine-1)/GDF-15 (growth differentiation factor-15) is expressed in a high proportion of melanoma cell lines compared to normal melanocytes and was detected in all examined metastatic melanoma biopsies (Boyle et al. 2009). GDF-15 levels are also increased five- to sixfold in sera from patients with an advanced melanoma compared to healthy donors (Huh et al. 2010). Affymetrix analysis of microdissected fresh frozen melanocytic nevi and melanoma samples showed upregulated expression of GDF-15 in primary and metastatic melanomas compared to melanocytic nevi (Mauerer et al. 2011). Interestingly, progression-free survival in melanoma patients with low GDF-15 staining was significantly higher compared to patients with high GDF-15 expression (Mauerer et al. 2011). Inhibition of GDF-15 expression with a specific shRNA strongly reduced tumor growth in an in vivo mouse xenograft model (Boyle et al. 2009; Huh et al. 2010). Inhibition of GDF-15 was found to delay melanoma tumor vascular development, subsequently affecting tumor cell proliferation and apoptosis. Melanoma cells secrete GDF-15 that promotes directional blood vessel development, in combination with VEGF (Huh et al. 2010). Expression of GDF-15 in melanoma cells is directly controlled by the transcription factor M-MITF (Boyle et al. 2009).

GDF-3/ Vgr-2 expression is also correlated with the metastatic capacity of melanoma cell lines and its overexpression promotes the growth of implanted melanoma tumors in a syngeneic mouse model. Moreover, GDF-3 expression is accompanied by an increased expression of CD24/CD44, markers of melanoma stem cell-like cells/melanoma-initiating cells (Ehira et al. 2010).

Together, these studies demonstrate the complexity of the TGF-β ligand/signaling network in melanoma and warrants further investigations.

10.7 Therapeutic Targeting of TGF-β to Fight Melanoma

There is broad experimental evidence for the potential benefit of targeting TGF-β signaling for cancer treatment. Different strategies have been developed to inhibit the TGF-β pathway: inhibition of TGF-β ligands expression with antisense molecules, blocking of ligands with ligand traps, and interference with receptor signaling by means of small molecules. These have been extensively reviewed over the last few years (Connolly et al. 2012; Hawinkels and Ten Dijke 2011; Wrzesinski et al. 2007).

For melanoma treatment, several anti-TGF-β therapies have been studied and they clearly present promising results. Antisense molecule targeting mRNA encoding the TGF-β2, called Trabedersen or AP12009, has been tested in clinical phase I/II study in patients with advanced pancreatic, malignant melanoma, or colorectal cancer. Traberdersen is safe and well tolerated and has shown promising efficacy. For instance, enrolled melanoma patients ($n = 14$) had reached a median overall survival of 9.3 months at the time of publication (Oettle et al. 2012).

Because TGF-β is abundantly secreted by tumor cells or by their microenvironment during disease progression, monoclonal TGF-β neutralizing antibodies and soluble TβRII or TβRIII have been elaborated to trap TGF-β ligands. In preclinical studies, this strategy has shown encouraging results in particular for breast cancer treatment (Connolly et al. 2012). An anti-TGF-β monoclonal neutralizing antibody, GC1008 (Fresolimumab) is under evaluation in a clinical phase I/II study in patients with advanced melanoma. In 2008, 5/22 patients with advanced melanoma achieved stable disease or partial responses. The most frequently reported drug-related side effects were skin rash/lesions including eruptive non-malignant keratoacanthomas and squamous cell carcinomas (Morris et al. 2008).

A number of small molecule inhibitors have been developed that target TGF-β receptor kinases. The majority of TβRI/ALK5 inhibitors also block the related activin and Nodal receptors ACVR1B/ALK4 and ACVR1C/ALK7, but with reduced affinity (Fu et al. 2008; Inman et al. 2002; Tojo et al. 2005). Several studies have illustrated the power of these inhibitors in the cancer treatment. As an example, in a mouse model of melanoma derived-bone metastasis, we have demonstrated that treatment with SD-208, a TβRI inhibitor, significantly reduces the incidence and size of osteolytic lesions in mice with established bone metastases compared to vehicle-treated mice (Mohammad et al. 2011). Unfortunately, a major drawback of these pharmacologic compounds is their high cardiotoxicity in humans, which has taken most of them out of clinical trials (Orphanos et al. 2009).

As discussed above, TGF-β plays a central role in allowing tumor immune escape. Some new antitumor strategies therefore consist in a combination of anti-TGF-β treatment and a vaccine, as blockade of TGF-β activity markedly enhances immunotherapy in preclinical models (Penafuerte and Galipeau 2008). It has been shown that TGF-β inhibits GM-CSF-induced maturation of bone marrow-derived dendritic cells, as well as expression of MHC class II and co-stimulatory molecules. Also, GM-CSF-secreting autologous immune vaccines have shown very good response in terms of immune stimulation and survival duration. The efficacy of these vaccines has been improved with a combination of TGF-β inhibition by means of TGF-β2 anti-sense oligonucleotides or specific shRNA targeting the proprotein convertase furin to prevent the conversion of latent TGF-β into an active growth factor (Olivares et al. 2011; Senzer et al. 2012).

High doses of IL-2 are a somewhat conventional treatment for metastatic melanoma in the USA despite limited response. Interestingly, nanoscale liposomal polymeric gel (nanolipogels) delivery of a TGF-β inhibitor together with IL-2 in a sustained fashion to the tumor microenvironment was found to significantly delay tumor growth and increase survival in tumor-bearing mice, accompanied with an increase in NK and TIL (tumor infiltrating lymphocytes) activity (Park et al. 2012).

10.8 Concluding Remarks

There is broad evidence underlying the involvement of TGF-β signaling in melanoma progression and metastasis development. TGF-β acts not only on the tumor cells but also on their microenvironment including stromal fibroblasts, endothelial and immune cells. Preclinical studies have reported very potent effects of anti-TGF-β approaches for cancer therapy. However, the effects of anti-TGF-β drugs have been less robust than hoped for in the clinical setting, one reason being their strong cardiotoxic activity. As numerous studies have exemplified the crosstalks between MEK/ERK and TGF-β pathways in epithelial cancers, it will be interesting to study the effect of TGF-β inhibitors in combination with the new melanoma drugs specifically targeting mutant BRAF or MEK. One may hope that combinatorial therapies will allow lower dosage to be used to circumvent drug toxicity and increase the clinical efficacy of TGF-β inhibitors.

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Chapter 11
The Transforming Growth Factor-Beta (TGF-β) in Liver Fibrosis

Isabel Fabregat and Patricia Sancho

Abstract Liver fibrosis is the final consequence of many chronic liver injuries that later develop in cirrhosis and hepatocellular carcinoma (HCC), which are leading causes of morbidity and mortality worldwide. The transforming growth factor-beta (TGF-β) represents a key cytokine that increases in liver in its activated form upon damage and triggers important cellular events during any progression stage of the disease. TGF-β mediates activation of hepatic stellate cells (HSCs) to myofibroblasts and induces cell death and epithelial mesenchymal transition (EMT) of hepatocytes. Both processes may facilitate extracellular matrix (ECM) deposition and scar formation. Regulatory T cells, important negative regulators of inflammation, depend on TGF-β for terminal differentiation, indicating its impact in the inflammatory response. Oxidative stress plays an essential role in mediating liver fibrosis, and recent studies demonstrate that TGF-β contributes to the reactive oxygen species (ROS) production and oxidative damage. Indeed, the active implication of TGF-β signaling in the progression of liver fibrosis makes this cytokine an attractive therapeutic target. In addition to the increasing number of compounds aimed at direct inhibition of the TGF-β pathway, the recent discovery of new downstream molecules with crucial roles in liver fibrosis development, such as NADPH oxidases, is opening the therapeutic perspectives.

Keywords Cell death • Chronic injury • Epithelial mesenchymal transition (EMT) • Hepatic stellate cell (HSC) • Hepatocyte • Inflammation • Myofibroblast • NADPH oxidases (NOX) • ROS

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Abbreviations

ALK5	Activin receptor-like kinase 5
CLD	Chronic liver disease
ECM	Extracellular matrix
EMT	Epithelial mesenchymal transition
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HSCs	Hepatic stellate cells
NASH	Non-alcoholic steatohepatitis
NOX	NADPH oxidase
ROS	Reactive oxygen species

11.1 Introduction

Liver fibrosis is the final consequence of many chronic liver injuries (Brenner 2009) that later develop in cirrhosis and hepatocellular carcinoma (HCC), which are leading causes of morbidity and mortality worldwide. The main etiologies of chronic liver diseases in industrialized countries include chronic hepatitis C virus (HCV) infection, alcohol, and non-alcoholic steatohepatitis (NASH). Regardless of the etiology, all the chronic liver diseases follow a common course: from middle inflammation, to more severe inflammation, to fibrosis and finally to cirrhosis. A complex and multistep process is involved in the progression to a chronic liver injury, which is evidenced by intracellular signal transduction changes, alteration in cell–cell and cell–extracellular matrix contacts and a drastic transdifferentiation of different cell types. During many years, research has been focused on the dissection of these pathways to develop new therapeutic approaches.

One of the cytokines whose levels increase in any kind of chronic liver disease (CLD) is the transforming growth factor-beta (TGF-β), which triggers important cellular events related to fibrogenesis and repair (Dooley and ten Dijke 2012; Hayashi and Sakai 2012). Most liver cells are sensitive to TGF-β, inducing both the canonical Smad-mediated and the non-canonical Smad-independent downstream signals. During the development of fibrosis, hepatic stellate cells (HSCs) respond to TGF-β moving to a myofibroblast phenotype, which in turn produces the higher deposition of extracellular matrix (ECM) proteins. TGF-β also plays essential roles during the inflammatory process linked to liver fibrosis, since it mediates the terminal differentiation of regulatory T cells, important negative regulators of inflammation. TGF-β induces cell death and epithelial mesenchymal transition of hepatocytes, and recent evidences indicate that this process might also contribute to the ECM deposition and scar formation. Activation of liver sinusoidal endothelial cells and

neoangiogenesis is also partially facilitated by TGF-β. Finally, TGF-β contributes to the reactive oxygen species (ROS) production and it is well known that oxidative stress plays an essential role in mediating liver fibrosis.

While the TGF-β role as "master" cytokine in chronic liver diseases is very clear, the complexity of the underlying response in cells and in the organ leading to the drastic changes observed is currently not fully understood. In this chapter, we will update the knowledge about the essential role of TGF-β in liver fibrosis, the proposed molecular mechanisms that mediate its actions, as well as new therapeutic approaches to inhibit its signaling.

11.2 Animal Models for the Study of Liver Fibrosis

During the last years, several mouse models of experimental fibrosis have been used for the study of the pathogenesis and molecular mechanisms associated with the diverse human pathologies leading to liver fibrosis. Among them, chemically induced fibrosis with hepatotoxic agents has been extensively investigated: thioacetamide, dimethylnitrosamine (DMN), and, most importantly, carbon tetrachloride (CCl_4), cause centrilobular parenchymal injury and fibrosis. These agents are processed by the cytochrome P-450 in hepatocytes, which releases damaging products causing massive hepatocyte cell death (Constandinou et al. 2005). In addition, concanavalin A is commonly used as a model for human chronic hepatitis since it triggers immune system-mediated fibrosis with similar histological characteristics (Louis et al. 2000). Finally, bile duct ligation constitutes a very used model for cholestatic fibrosis, triggering extrahepatic biliary atresia and primary sclerosing cholangitis (Constandinou et al. 2005). Most importantly, knockout mice have become a powerful strategy for the last years to study the molecular mechanisms of fibrosis, focusing on the contribution of one or more genes to the pathogenesis of the disease (Hayashi and Sakai 2011). These knockout mice, in addition, have helped to establish new genetic models of liver fibrosis such as the $Mdr2^{-/-}$ mouse, which develops spontaneous sclerosing cholangitis (Fickert et al. 2004).

Studies with transgenic mice that overexpress TGF-β have demonstrated that this cytokine alone is sufficient to induce fibrosis, independently of the primary cause of the disease. The hepatic expression of TGF-β induces upregulation of pro-collagen I and pro-collagen III mRNAs in the hepatic tissue, and deposition of extracellular matrix in the sinusoid (Kanzler et al. 2001). Similar results were obtained in a conditional tetracycline-regulated expression of TGF-β1 in liver of transgenic mice, where fibrosis progressed to an intermediary state (Ueberham et al. 2003). In both cases, activation of HSC was observed. Inversely, in experimental models of liver fibrosis, the fibrogenic process can be attenuated simply by blockade of TGF-β signaling (Ueno et al. 2000). These results together point out to the relevant role played by TGF-β in liver fibrogenesis.

11.3 Effects of TGF-β in Liver Cells: Relevance in Liver Fibrosis

The response to chronic liver injury involves different cell types and undergoes different phases (Dooley and ten Dijke 2012). Initially, liver injury induces epithelial cell stress, which causes cell death either through necrosis or apoptosis. Death-mediated signals and necrotic cells induce a strong inflammation and wound-healing response, as well as activation of HSC. These events may conduct to liver regeneration and repair, but acute setting addresses a fibrogenic process. Below we will discuss the role of TGF-β in these processes.

11.3.1 Role of TGF-β in the Activation of Hepatic Stellate Cells to Myofibroblasts

Regulation of extracellular matrix accumulation in acute and chronic liver injuries involves different mechanisms, but HSCs appear to be the principal effecter in all cases (Friedman 2010). The HSCs are the major storage site of retinoids in the body and are present in the space of Disse in close contact with hepatocytes and the sinusoid. When the HSC is activated, it loses its retinoid content, increases proliferation and motility, expresses new markers, such as smooth muscle actin, and produces ECM proteins. In the normal liver, sinusoidal endothelial cells and Kupffer cells (macrophages) contain relatively high levels of TGF-β mRNA, whereas HSCs express little amounts of TGF-β. However, in response to pro-fibrogenic stimuli HSCs express the three different isoforms of TGF-β and contribute to the development of fibrosis through both autocrine and paracrine loops of TGF-β-stimulated collagen production (Inagaki et al. 2005). The main cell responsible for the fibrosis is the myofibroblast, which produces the fibrous scar found in all chronic liver diseases. Different lines of evidence support the hypothesis that one of the main sources of these myofibroblasts are the quiescent HSC that become activated in response to TGF-β (Fig. 11.1): (1) downregulation of TGF-β expression in liver, by using adenoviruses or genetically modified animals, reveal a failure in HSC activation and fibrogenesis (Hellerbrand et al. 1999; Kanzler et al. 1999); (2) in vitro experiments reveal that HSCs are responsive to TGF-β treatment and transduce a signal that may play important roles in fibrogenesis (Dooley et al. 2000); (3) gene transfer of Smad7, the member of inhibitory Smads, inhibits experimental fibrogenesis, which is coincident with arrested transdifferentiation of primary cultured HSCs to myofibroblasts (Dooley et al. 2003). The response of HSCs to TGF-β, leading to, e.g., induction of α2 (I) collagen expression, is mediated by phosphorylation of Smad2 and Smad3 and subsequent nuclear translocation of a Smad-containing complex (Dooley et al. 2001). Maximal expression of collagen type I in activated HSCs requires Smad3 in vivo and in culture (Schnabl et al. 2001). Interestingly, Smad3 is not necessary for HSC activation as assessed by alpha-SMA expression, but is necessary for inhibition

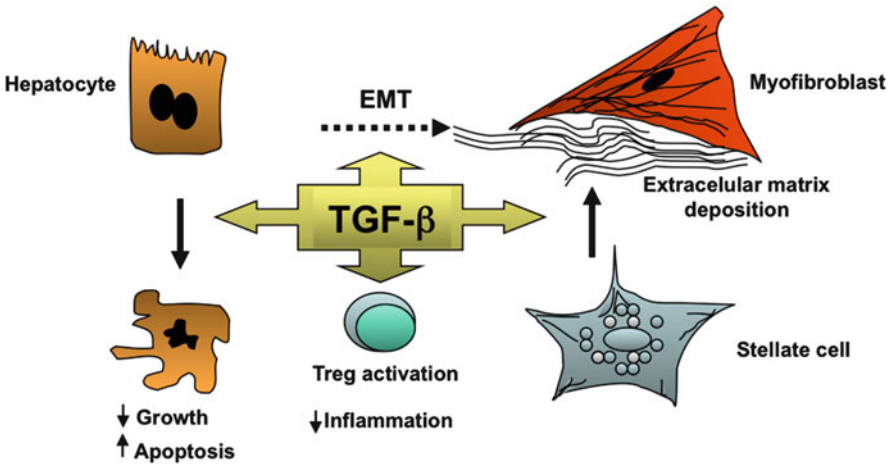

Fig. 11.1 Effects of TGF-β in liver and inflammatory cells. *Effects that may counteract liver fibrosis*: (1) TGF-β triggers activation of hepatic stellate cells to myofibroblats, which are considered the main producers of extracellular matrix proteins. (2) TGF-β induces growth inhibition and cell death of hepatocytes, which impair liver regeneration. (3) The hepatocytes that survive to the inhibitory signals may respond to TGF-β undergoing epithelial mesenchymal transition (EMT). Although controversial, different reports indicate that this process exists and would facilitate ECM deposition and scar formation. *Effects that may counteract liver fibrosis*: regulatory T cells, important negative regulators of inflammation, depend on TGF-β for terminal differentiation, which would have beneficial consequences impairing the fibrotic process

of proliferation of HSCs, which is TGF-β-dependent, and is required for TGF-β1-mediated Smad-containing DNA-binding complex formation in cultured HSCs. These data indicate that HSCs are responsive to TGF-β treatment and transduce a signal that may play an important role in liver fibrogenesis. Myofibroblasts display decreased availability of surface receptors for TGF-β, which could be based on autocrine stimulation. However, lack of activated Smad complexes with DNA-binding activity and absence of α2 (I) collagen transcription inhibition by latency-associated peptide (LAP)/anti-TGF-β antibody raise the possibility of TGF-β signaling independent receptor downregulation in myofibroblasts (Dooley et al. 2000).

11.3.2 Role of TGF-β on Hepatocytes: Relevance in Liver Fibrosis

Chronic liver injuries are characterized by persistent hepatocyte damage and death, induced by chemical toxicity, metabolic overload, viral/microbial infections, etc., which cause metabolic deregulation and oxidative stress. Several modes of cell death have been classified in the damaged liver, including apoptosis and necrosis.

It is now fully accepted that hepatocyte death is critical for hepatic fibrosis (Brenner 2009; Nikolaou et al. 2012). It appears that the primary response to injury would be liver regeneration, but if it is blocked, the default mode will be liver fibrosis. If hepatocytes undergo apoptosis without compensatory proliferation, fibrosis again would result. Indeed, it has been proven that apoptosis and phagocytosis of hepatocytes directly induce HSC activation and initiation of fibrosis (Jiang et al. 2010), and hepatocyte apoptotic bodies during chronic hepatitis C infection amplify stellate cell activation. TGF-β1 might be involved in the impairment of liver regeneration and in amplifying hepatocyte apoptosis (Fig. 11.1). Indeed, TGF-β is an important regulatory suppressor factor in hepatocytes, inhibiting proliferation (Carr et al. 1986) and inducing cell death (Oberhammer et al. 1992). The increase in TGF-β levels in the first stages of liver fibrogenesis may be responsible for an imbalance in the proliferative and survival signals in hepatocytes, contributing to the failure in liver regeneration.

However, paradoxically, in addition to its suppressor effects, TGF-β also induces anti-apoptotic signals in proliferating hepatocytes and hepatoma cells (Valdes et al. 2004; Caja et al. 2007), through activation of the epidermal growth receptor (EGFR) pathway (Murillo et al. 2005). Cells that survive to TGF-β-induced apoptotic signals undergo epithelial mesenchymal transition (EMT) (Gotzmann et al. 2002; Valdes et al. 2002; Caja et al. 2007; 2011; Kaimori et al. 2007), a physiological process during embryogenesis, in which an epithelial cell loses expression of adhesion molecules, such as E-cadherin, and other components responsible for cell polarity. Instead, they express mesenchymal components of cytoskeleton and acquire motility and scattering properties (Thiery et al. 2009). Certain evidences indicate that a crosstalk exists between the genetic programs that control TGF-β-induced growth arrest/apoptosis and those that regulate EMT. Indeed, once hepatocytes undergo EMT they become resistant to TGF-β-induced apoptosis (Valdes et al. 2002), a process in which transcription factors of the Snail family, repressors of the E-cadherin gene, are involved (Franco et al. 2010). A closely related phenotypic conversion is also detected in some models of fibrosis and may be associated with disease progression (Lopez-Novoa and Nieto 2009). In the case of the liver, the role of EMT from hepatocytes to myofibroblasts is perhaps the most intriguing and controversial of recent hypotheses on the origin mechanisms of liver fibrosis (Wells 2011). Strong evidences indicate that hepatocytes from transgenic animals overexpressing Snail (a master gene involved in EMT through its capacity to repress E-cadherin gene, among others) fully undergo EMT (Franco et al. 2010) and might propagate liver fibrosis progression (Rowe et al. 2011). However, under normal genetic background, data from different experimental approaches in animals and humans show controversy. Some reports support a role for EMT in epithelial cells in the liver that might transform into myofibroblasts (Zeisberg et al. 2007; Dooley et al. 2008), whereas others show no evidence of EMT in models of hepatic fibrosis (Taura et al. 2010; Chu et al. 2011). Further experiments are required to fully conclude that TGF-β plays a role in transdifferentiation of hepatocytes to myofibroblasts through EMT processes (Fig. 11.1).

11.4 Crosstalk Between TGF-β and Inflammatory Signals

Inflammation plays an essential role in the development of liver fibrosis. When a chronic injury takes place, a large infiltration of mononuclear cells, which include macrophages, lymphocytes, eosinophils, and plasma cells, occur. Mobilization of lymphocytes produces lymphokines that activate macrophages, which, in turn, stimulate lymphocytes, fibroblasts, and other inflammatory cells, thus setting the stage for persistence of an inflammatory response (Wynn and Barron 2010). Furthermore, macrophages produce pro-fibrotic mediators, including TGF-β1 and PDGF, and control extracellular matrix turnover by regulating the balance of various matrix metalloproteases and tissue inhibitors of metalloproteases. Examples of knockout mice that are resistant to fibrosis because they have less inflammation include those with gene deletions of TNF-α or Toll-like receptor 4 (TLR4), among others (Kitamura et al. 2002; Seki et al. 2007).

Crosstalk between TGF-β and inflammatory signals occurs at different levels. On one side, from studies in different tissues including the liver, TGF-β is believed to play an important role in the regulation of the immune system. Indeed, it activates the differentiation of regulatory T cells (Treg) (Hammerich et al. 2011), a unique subset of $CD4^+$ T-helper cells that control effector T-cell responses to prevent autoimmune reactions. Activated Treg produce the anti-inflammatory cytokine IL-10, which would have beneficial effects in a pro-fibrotic process (Fig. 11.1). However, on the other side, perturbation of TGF-β signaling by pro-inflammatory cytokines in liver cells contributes to both fibrogenesis and carcinogenesis (fibro-carcinogenesis). Smad proteins have intermediate linker regions between conserved Mad homology (MH) 1 and MH2 domains. TGF-β type I receptor and pro-inflammatory cytokine-activated kinases differentially phosphorylate Smad2 and Smad3 to create phosphoisoforms that are phosphorylated at the COOH-terminal (C), linker (L), or both (L/C) regions (Matsuzaki 2009). TGF-β and pro-inflammatory cytokines synergistically enhance collagen synthesis by activated hepatic stellate cells via pSmad2L/C and pSmad3L/C pathways. During chronic liver disease progression, pre-neoplastic hepatocytes persistently affected by TGF-β together with pro-inflammatory cytokines come to exhibit the same carcinogenic (mitogenic) pSmad3L and fibrogenic pSmad2L/C signaling as do myofibroblasts, thereby accelerating liver fibrosis while increasing risk of HCC (Matsuzaki 2009). c-Jun N-terminal kinase (JNK) activated by pro-inflammatory cytokines is mediating this perturbed hepatocytic TGF-β signaling (Yoshida et al. 2005). Under normal conditions, to avoid unlimited extracellular matrix deposition, Smad7 induced by TGF-β negatively regulates its pro-fibrogenic response. In the presence of pro-inflammatory cytokines and activation of the JNK and MAPKs pathways, Smad7 cannot be induced by the pSmad3L pathway (Yoshida and Matsuzaki 2012). Another example of modulation of TGF-β signals by pro-inflammatory cytokines comes from studies in the TLR4-chimeric mice (Seki et al. 2007). In quiescent HSCs, TLR4 activation not only upregulates chemokine secretion and induces chemotaxis of Kupffer cells, but also downregulates the TGF-β pseudoreceptor Bambi, to sensitize HSCs to TGF-β-induced signals

and allow unrestricted activation by Kupffer cells. Clinical relevance of the crosstalk between TLR4 and the TGF-β/Bambi signaling has been demonstrated in studies of liver fibrosis progression in hepatitis C and hypercholesterolemic patients (Guo et al. 2009; Teratani et al. 2012). Finally, there is evidence that Th2 cytokines cooperate with TGF-β to induce fibrosis (Wynn 2008). IL-13 activates the production of latent TGF-β in macrophages and upregulates the expression of proteins that cleave the Latent Association Protein (LAP), which contributes to the release of active TGF-β (Lee et al. 2001).

11.5 Reactive Oxygen Species in Liver Fibrosis: Connection with the TGF-β Pathway

ROS, including H_2O_2, OH·, and O_2^-, are critical intermediates in both the normal physiology and pathological conditions of liver cells. When the equilibrium between ROS generation and the antioxidant defense of the cell is disrupted, it results in an oxidative stress process (Sies and Cadenas 1985). As commented above, fibrosis has been well documented in many chronic liver diseases, usually beginning with an inflammatory phase which progresses to fibrosis after chronic oxidative stress (Diesen and Kuo 2010). ROS play a central role in the development of liver fibrosis/cirrhosis by both alcohol and hepatitis virus core proteins (Perlemuter et al. 2003; Dionisio et al. 2009). In addition, oxidative stress markers have been detected in the serum of and biopsy samples from liver cirrhosis patients and in experimental liver fibrosis/cirrhosis animals (Yadav et al. 2002; Pawlak et al. 2008). Moreover, in liver biopsies, areas of fibrosis were localized to areas with increased 4-hydroxy-2′-nonenal (4-HNE), a marker of lipid peroxidation (MacDonald et al. 2001; Seki et al. 2005).

In relation to TGF-β, ROS play a complex role promoting fibrosis progression. On one side, they constitute a commonly known downstream effector implicated in TGF-β signaling (Liu and Gaston Pravia 2010). On the other side, ROS may also promote fibrosis activating latent TGF-β through either LAP direct oxidation and subsequent release of the cytokine (Pociask et al. 2004) or via MMP activation (Wang et al. 2005). Indeed, LAP/TGF-β1 complex has been proposed to function as an oxidative stress sensor (Jobling et al. 2006). Finally, ROS can also stimulate the expression and secretion of TGF-β in a positive feedback loop in many types of cells, including hepatic stellate cells and hepatocytes (Proell et al. 2007; Boudreau et al. 2009).

11.5.1 Subcellular Sources of ROS in Liver Fibrosis

The primary cellular sources of oxidative stress during the inflammatory phase of liver fibrosis are mainly neutrophils, Kupffer cells, and, specially, hepatocytes. Although for many years the mitochondria have been considered as the major

source for ROS in the living cells, we have to consider two additional ROS-producing systems playing determinant roles in the liver pathophysiology, such as the P450 system in hepatocytes and the NADPH oxidases (NOX) proteins in different liver cells.

Mitochondria play a central role for ROS production in the liver, since hepatocytes contain hundreds of these organelles and the mitochondrial electron transport is disrupted in a great number of pathophysiological circumstances, resulting in increased electron leak (Murphy 2009). Indeed, several reports have suggested a central role for mitochondrial ROS in hepatic toxicity in models of hepatic cholestasis (Graf et al. 2002; Fang et al. 2004) and alcoholic disease (Kukielka et al. 1994; Zhu et al. 2012). Most importantly, several reports have shown that direct treatment with TGF-β induces a prolonged mitochondrial ROS production in rat hepatocytes (Albright et al. 2003; Herrera et al. 2004). This fact can be attributed to its capacity of downregulating the expression of several antioxidant enzymes, such as glutaredoxin, catalase, superoxide dismutase, and glutathione peroxidase (GPx) (Franklin et al. 2003; Herrera et al. 2004).

CYP2E1, the hepatocytic member of the cytochrome P450 oxidase system, is involved in the metabolism of xenobiotics in the body. Most drugs and hepatotoxins are detoxified by CYP2E1, which can generate ROS as a byproduct of the oxidative reaction. Both in vitro experiments and animal studies in vivo have demonstrated that CYP2E1 is an important source of ROS in alcohol-induced liver injury, and its expression is inducible by alcohol (Zhu et al. 2012). Importantly, it has been reported that TGF-β enhances hepatocyte toxicity in cells overexpressing CYP2E1 upon ethanol exposure (Zhuge and Cederbaum 2006).

Other main source of ROS implicated in TGF-β signaling and fibrosis is the NOX family of NADPH oxidases. This family has been discovered for homology to $gp91^{phox}$, the phagocytic oxidase. Nowadays, the NOX family includes seven different members NOX1 to NOX5, DUOX1, and DUOX2 (Bedard and Krause 2007) whose main function is active ROS production. NOX proteins have been previously related to fibrosis in several organs such as lung (Hecker et al. 2009), pancreas (Masamune et al. 2008), kidney (Sedeek et al. 2010), and heart (Cucoranu et al. 2005). In the liver, several reports have demonstrated a key role for NOX proteins in the progression of hepatic fibrosis (De Minicis et al. 2010; Cui et al. 2011; Paik et al. 2011; Jiang et al. 2012; Sancho et al. 2012). The isoforms expressed by the different resident populations of the liver are mainly NOX1, NOX2, and NOX4 (Paik et al. 2011).

11.5.2 Implication of ROS in the Molecular Mechanisms Mediating Liver Fibrosis

One of the most studied mechanisms of fibrogenesis actually influenced by ROS is myofibroblast activation. In the liver, stellate cell transdifferentiation into myofibroblast is inhibited by antioxidants (Foo et al. 2011; Abhilash et al. 2012). Indeed,

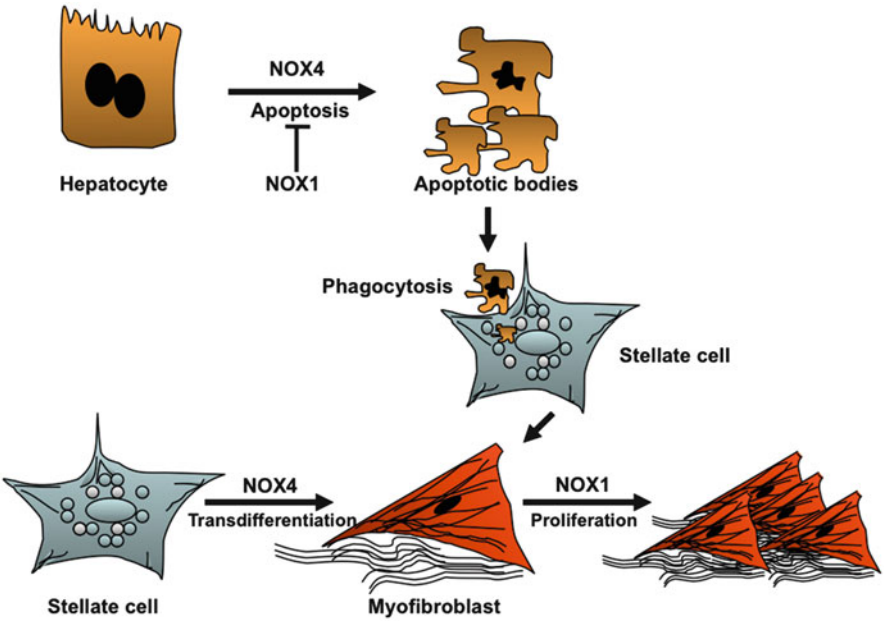

Fig. 11.2 NOX proteins play crucial different roles during liver fibrosis development. Opposite functions of NOX1 and NOX4 in hepatocytes: NOX1 protects cells from pro-apoptotic stimuli and mediates proliferation, while NOX4 promotes TGF-β-induced cell death. Afterwards, stellate cells can phagocytose the resulting apoptotic bodies, which functions as a triggering signal for activation. Primed stellate cells can also suffer transdifferentiation into myofibroblasts in response to TGF-β, a process where NOX4 plays a determinant role controlling the acquisition of the activated phenotype. Finally, and once fully activated, NOX1 favors myofibroblast proliferation, contributing to fibrosis development

NOX4 downstream TGF-β has been described as the main mediator for myofibroblast activation in different organs such as heart (Cucoranu et al. 2005), lung (Hecker et al. 2009), kidney (Bondi et al. 2010), and diseased prostatic stroma (Sampson et al. 2011). Equivalently, it has been demonstrated in cultured HSC that TGF-β-induced transdifferentiation is accompanied by NOX4-derived ROS (Proell et al. 2007), which can be a useful target for therapeutic approaches (Ikeda et al. 2011). Very recently, two different reports have described a key role for NOX4 in hepatic myofibroblasts activation downstream TGF-β (Jiang et al. 2012; Sancho et al. 2012) both in vivo and in vitro (Fig. 11.2). In these works, HSC activation was attenuated either by NOX4 downregulation or in a $Nox4^{-/-}$ genetic background, and, importantly, the myofibroblast-activated state could be also reversed by NOX4 downregulation (Sancho et al. 2012). However, the role of NOX proteins in liver fibrogenesis is not only circumscribed to NOX4. Thus, studies performed in $Nox1^{-/-}$, $Nox2^{-/-}$, or $p47phox^{-/-}$ mice have pointed out the importance of NOX1 and NOX2 in fibrosis development (De Minicis et al. 2010; Jiang et al. 2010; Cui et al. 2011; Paik et al. 2011). Concretely, NOX1 promotes myofibroblast proliferation by PTEN

inactivation to positively regulate an Akt/FOXO4/p27 signaling pathway (Cui et al. 2011). Indeed, NOX1 seems to mediate the pro-fibrogenic effects exclusively in endogenous liver cells, while NOX2 could be implicated in both endogenous liver cells and bone marrow-derived cells (Paik et al. 2011), possibly acting in the process of phagocytosis of dead hepatocytes (Jiang et al. 2010) (Fig. 11.2).

Finally, promotion of hepatocyte apoptosis constitutes also a crucial mechanism influenced by TGF-β-induced ROS. In fact, and as mentioned before, TGF-β induces apoptosis through ROS that is derived from both mitochondria and NOX activity (Herrera et al. 2004). Indeed, pretreatment with antioxidants block apoptosis (Sanchez et al. 1996; Herrera et al. 2001). Recently, it has been described that hepatocytes express different members of the NOX family, mainly NOX1, NOX2, and NOX4 (Murillo et al. 2007), which play opposite roles in the control of hepatocyte survival and death. Indeed, NOX4 is necessary to mediate apoptosis induced by TGF-β (Carmona-Cuenca et al. 2008; Caja et al. 2009), but the pro-apoptotic effect of the cytokine can be attenuated when NOX1 is active (Sancho et al. 2009; Sancho and Fabregat 2011; Ortiz et al. 2012) (Fig. 11.2). In addition, $Nox4^{-/-}$ hepatocytes are also resistant to apoptosis induction by other stimuli, such as FasL and TNF-α/actinomycin D (Jiang et al. 2012). In addition, NOX1 activity might further contribute to the inflammatory process promoting COX-2 expression and prostaglandin synthesis in hepatocytes (Sancho et al. 2011). Interestingly, dual NOX4/NOX1 pharmacological inhibition with GKT137831 is able to diminish both the apparition of fibrogenic markers and hepatocyte apoptosis in vivo upon bile duct ligation (Jiang et al. 2012), reinforcing the relevant role of NOX1 and NOX4 in liver fibrosis and opening new perspectives for its treatment.

11.6 TGF-β Pathway Inhibitors as a Promising Therapy in Liver Fibrosis

During the last years, after the role of TGF-β signaling in cancer and other pathologies, including fibrosis, became established, a great effort has been made in order to develop different approaches to inhibit TGF-β pathway. Thus, the number of possible compounds used either in preclinical or clinical studies related to fibrosis is continuously growing, thanks to previous experiences in other pathologies. The different strategies to block the TGF-β pathway can be classified as: (1) ligand traps, which include blocking antibodies and inhibitory peptides; (2) antisense oligos; (3) receptor kinase inhibitors; (4) Smad inhibitors; and (5) indirect inhibitors (Table 11.1). However, the list of compounds tested for liver fibrosis is rather reduced when compared with all the available inhibitors, since clinical efforts have concentrated for the last few years in blocking the underlying pathology specific for each type of fibrosis.

One of the most studied strategies for inhibiting the TGF-β pathway related to liver fibrosis is the ligand trapping, either by soluble receptors or inhibitory peptides. Indeed, several studies have demonstrated the antifibrotic potential of a

Table 11.1 Current preclinical and clinical TGF-β-based therapeutic strategies

Class	Drug	Target	Disease	References/Trial ID
Blocking antibodies	CAT-192	TGF-β_1	Systemic sclerosis	NCT00043706
			Myelofibrosis	NTC01291784
	CAT-152	TGF-β_2	Trabeculectomy, renal fibrosis	Hill et al. (2001), Grehn et al. (2007), Khaw et al. (2007)
	LY238770	TGF-β_1	Diabetes	NTC01113801
	GC1008	Pan-TGF-β	Systemic sclerosis	NCT01284322
			Myelofibrosis	NCT01291784
			Idiopathic pulmonary fibrosis	NCT00125385
			Glomerulosclerosis (FSGS)	NCT00464321
Peptide inhibitor	sTβRII	Pan-TGF-β	Liver fibrosis	Sullivan et al. (2010), Yao et al. (2010), Nakamuta et al. (2005), Cui et al. (2003), Yata et al. (2002), Ueno et al. (2000), George et al. (1999)
	P144	TGF-β_1	Skin fibrosis	NCT00781053
			Myocardial and liver fibrosis	Ezquerro et al. (2003), Hermida et al. (2009)
Kinase inhibitors	GW388788	TβRII/ALK5	Infarction, renal and skin fibrosis	Lagares et al. (2010), Petersen et al. (2008), Tan et al. (2010)
	SKI2162	ALK5	Peyronie's disease	Piao et al. (2010)
	GW6604	ALK5	Liver fibrosis	De Gouville et al. (2005)
	LY2109761	TβRII/ALK5	Several cancers, pulmonary fibrosis	Connolly et al. (2011), Fransvea et al. (2008), Flechsig et al. (2012), Ganapathy et al. (2010), Lacher et al. (2006), Zhang et al. (2010, 2011)
	SD208	ALK5	Scleroderma	Chen et al. (2006)
	SM16	ALK5	Vascular fibrosis	Fu et al. (2008)
	IN-1130	ALK5	Renal fibrosis, Peyronie's disease	Moon et al. (2006), Ryu et al. (2009)
Smad inhibitors	HSc025	Smad-dependent transcription	Systemic sclerosis	Hasegawa et al. (2009)
			Liver fibrosis	Higashi et al. (2011)
	SiS3	Smad3	Liver fibrosis	Matsubara et al. (2011)

(continued)

Table 11.1 (continued)

Class	Drug	Target	Disease	References/Trial ID
Indirect inhibitors	STX-100	αvβ6 integrin	Idiopathic pulmonary fibrosis	NCT01371305
	Tranilast	Not known	Rheumatoid arthritis	NCT00882024
			Lupus (LMDF)	Koike et al. (2011)
			Crohn's disease	Oshitani et al. (2007)
			Diabetes	Martin et al. (2005), Kelly et al. (2007)
			Hypertension	Hocher et al. (2002), Kagitani et al. (2004)
			Liver fibrosis	Ikeda et al. (1996), Uno et al. (2008), Said et al. (2012)

soluble type II receptor. This antagonist, consisting of a chimeric IgG containing the extracellular portion of the TGF-β type II receptor, was able to inhibit several fibrosis markers when tested in the model of bile duct ligation in mice (George et al. 1999). Importantly, it also showed to be dose-dependently effective in models of chemical liver fibrosis with carbon tetrachloride or DMN at either short- or long-term evaluation (Yata et al. 2002; Nakamuta et al. 2005). Interestingly, in this last publication authors demonstrated that remote delivery of the compound in the muscle is effective in inhibiting hepatic alterations. Recently, effectiveness of this compound has been improved using a novel strategy consisting of a fusion protein formed by the soluble portion of TβRII and IFN-γ administered intraperitoneally in a model of experimental fibrosis in rats (Yao et al. 2010). Alternatively, several studies have exploited a genetic approach using adenovirus containing the ectodomain of the TβRII alone or fused with other proteins. This approach was tested in vitro by infection of primary rat HSC, showing an inhibitory effect on the autocrine TGF-β production concomitant with transdifferentiation into myofibroblasts (Cui et al. 2003). In addition, the adenovirus strategy has shown to be effective in vivo with no apparent side effects. In this case, adenovirus expressing the entire ectodomain of the human TβRII fused to the Fc portion of human IgG (AdTbeta-ExR) was injected in the skeletal muscle in rats (Ueno et al. 2000).

Additionally, a soluble form of the type III receptor (betaglycan) has also been tested both at the preclinical and at the clinical levels for treatment of different fibrosis-related diseases, such as pulmonary, cardiac, and skin fibrosis (Liu et al. 2002; Hermida et al. 2009). Regarding the liver, a short peptide derived from this receptor, P144, showed in vitro efficacy blocking TGF-β-dependent stimulation of the human α2(I) collagen promoter. Importantly, intraperitoneal administration of P144 was able to diminish histological fibrosis markers and the number of

myofibroblats in rats treated with carbon tetrachloride, with no apparent side effects (Ezquerro et al. 2003).

Although inhibiting the kinase activity of TGF-β receptors is clearly the most recurrent strategy used in current preclinical and clinical therapies related to this cytokine, no significant studies referring to liver fibrosis have been performed. However, a great number of inhibitors of the ATP binding site of TβRI kinase (activin receptor-like kinase 5: ALK5) have been designed and preclinically tested in various fibrosis-related diseases and, may be eventually, used for liver fibrosis treatment. Up to date, only one kinase inhibitor, GW6604, has shown beneficial effects preventing liver damage in both an acute model of liver disease and a chronic model of dimethylnitrosamine (DMN)-induced liver fibrosis (de Gouville et al. 2005). In this last chronic model, where DMN was administered for 6 weeks and GW6604 dosed for the last 3 weeks, mortality was prevented, which correlated with reduced matrix deposition and decreased liver function deterioration. Another compound, IN-1130, has also potential applicability for liver fibrosis treatment. Indeed, this compound, which has been positively tested for renal fibrosis and Peyronie's disease (Moon et al. 2006; Ryu et al. 2009), is preferentially accumulated in the liver upon oral administration (Kim et al. 2008).

Alternatively, new compounds have been recently discovered which act by inhibiting Smad-dependent transcriptional inhibition. The indol-derivative SiS3 is a specific inhibitor of Smad3 phosphorylation and activity, and it was first described to be effective in inhibiting the activated phenotype of scleroderma fibroblast (Jinnin et al. 2006). Regarding liver fibrosis, only one in vitro preclinical study using a cholestatic disease model has been published so far. In this work, SiS3 treatment was able to inhibit the expression of several genes related to cholestasis development (Matsubara et al. 2011). In addition, the compound Hsc025 is a Smad-dependent transcriptional inhibitor that has been effectively tested in mice models of skin, pulmonary and hepatic fibrosis by oral administration (Hasegawa et al. 2009; Higashi et al. 2011). Indeed, in vitro treatment with HSc025 significantly suppressed collagen gene expression in cultured HSC, while oral administration of HSc025 improved liver injury and hepatic fibrosis degree in mice treated with carbon tetrachloride (Higashi et al. 2011).

Although its effects at the molecular level are not fully understood, tranilast is a drug mainly described as a collagen expression inhibitor, thus possessing antifibrotic properties. Indeed, while it can also inhibit the production of other cytokines, the major effect described for tranilast is the inhibition of both TGF-β expression and action (Miyazawa et al. 1995; Ikeda et al. 1996; Platten et al. 2001). This compound showed potential for hepatic fibrosis treatment several years ago, when it was described that tranilast treatment inhibited the expression of pro-collagen and TGF--β (Ikeda et al. 1996). Importantly, this compound also was effective in two different in vivo models of liver fibrosis. First, using a dietary model of NASH where obese diabetic and nondiabetic rats were fed with a methionine-deficient and choline-deficient diet, treatment with tranilast was effective at two different levels (Uno et al. 2008): on one side, it was able to inhibit fibrosis development and the activation of stellate cells, downregulating the expression of TGF-β, pro-collagen, and

plasminogen activator-1; on the other side, it attenuated hepatic inflammation and Kupffer cell recruitment, downregulating the expression of TNFα. In the second model, tranilast was able to improve hepatic fibrosis due to schistosomal infection, proved by a significant improvement of hepatic functions, reduction of the histopathological changes and collagen content, and decreased TGF-β1 levels in serum (Said et al. 2012).

11.7 Conclusions

Liver fibrosis is one of the main causes of mortality worldwide. Nowadays, a lot of effort is being made in order to increase the knowledge of the molecular mechanisms underlying this complicated disease, in which TGF-β seems to play a determinant role. Indeed, the active implication of TGF-β signaling in the progression of liver fibrosis, regardless of its original etiology, makes this cytokine an attractive therapeutic target for the development of new treatments. In addition to the increasing number of compounds aimed at direct inhibition of the TGF-β pathway, the recent discovery of new downstream molecules with crucial roles in liver fibrosis development, such as NADPH oxidases, is opening the therapeutic perspectives. Indeed, specific targeting of these molecules could be an important step forward in the treatment of the disease, since its inhibition may be effective enough avoiding the possible side effects of TGF-β systemic inhibition.

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Chapter 12
TGF-β and Inhibitory Smads in Inflammation

Seong-Jin Kim and Seok Hee Park

Abstract TGF-β is a multifunctional cytokine involved in diverse cellular functions, including cell growth, apoptosis, and immune responses, although its effect is likely to be dependent on cell context. Among a variety of cellular functions exerted by TGF-β, recent advances emphasize the importance of TGF-β and its signaling pathway in innate immunity and adaptive immunity. The typical examples of immune regulations by TGF-β include suppression of toll-like receptor (TLR) signaling which recognizes the invading pathogens and T cell differentiations such as Th17 and Treg. In particular, much attention has been paid to anti-inflammatory function of TGF-β, which is mediated by the inhibitory Smads, Smad6 and Smad7. In this review, we mainly discuss the anti-inflammatory role of TGF-β suppressing inflammatory responses and the underlying mechanism mediated by the inhibitory Smads.

Keywords Inflammation • Inhibitory Smads • Innate immune responses • Smad6 • Smad7

12.1 Introduction

Although extensive studies have been performed to reveal essential roles of TGF-β and the related molecular mechanism during the past 25 years (Blobe et al. 2000; Derynck and Zhang 2003; Heldin et al. 1997; Letterio and Roberts 1997; Massague 1998; Miyazono et al. 2000), the functions of TGF-β and its signaling pathway are

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very complicated and controversial. The major reason is that TGF-β signaling pathways are involved in most cellular functions showing cell context-dependent fashion and forming highly intricate signaling networks through cross talking with other cellular signaling pathways.

There are three TGF-β members (TGF-β1, TGF-β2, and TGF-β3) present in mammals. Among them, TGF-β1 is the major form found in the immune system. TGF-β signaling pathways are mainly divided into canonical and noncanonical pathways (Derynck and Zhang 2003; Mu et al. 2012). TGF-β canonical pathway is mediated by heterodimeric receptors with serine/threonine receptor kinases and cytoplasmic proteins called Smads. Upon binding to TGF-β, type II (TβRII) and type I (TβRI) receptors initiate the signaling process with their intrinsic kinase activities, and then the activated type I receptor transmits intracellular signals through the phosphorylation of receptor-activated Smad proteins (R-Smads), Smad2 and Smad3. The phosphorylated R-Smads form heteromeric complexes with common partner Smad4 (Co-Smad) and translocate into the nucleus to modulate expressions of diverse target genes (Derynck and Zhang 2003; Massague and Wotton 2000; Miyazono et al. 2000). The more interesting finding is that this canonical TGF-β signaling has its own regulatory system, called inhibitory Smads (I-Smads). The I-Smads include Smad7 and Smad6 proteins (Hayashi et al. 1997; Imamura et al. 1997; Nakao et al. 1997). Smad7 is transcriptionally induced by TGF-β and acts as an intracellular antagonist through stable interaction of activated TβRI receptor, resulting in the formation of autoregulatory negative feedback loop of TGF-β signaling (Hayashi et al. 1997; Nakao et al. 1997). Smad6, another inhibitory Smad, is also induced by TGF-β in several cell lines such as mink lung epithelial (Mv1Lu) cells, human keratinocyte (HaCaT) cells, CMT-93 mouse intestinal epithelial cells, and mouse primary peritoneal macrophages (Afrakhte et al. 1998; Choi et al. 2006). It has been initially identified as an inhibitor of Smad2 and Smad1 phosphorylation by TGF-β superfamily (Imamura et al. 1997; Ishida et al. 2000), and it is likely to be an important regulator involved in TGF-β signaling (Afrakhte et al. 1998; Choi et al. 2006; Kleeff et al. 1999).

In contrast, TGF-β noncanonical signaling is independent of the activations of R-Smads, and TGF-β utilizes a variety of cellular signaling pathways such as p38 and JNK mitogen-activated protein kinases (MAPK), PI3 kinase, and TRAF6-TAK1 pathways (Mu et al. 2012; Zhang 2009). Therefore, TGF-β noncanonical pathways complicate the understanding of the precise molecular mechanisms underlying TGF-β-mediated cellular functions.

Inflammation is defined as a protective response to eliminate detrimental stimuli and to repair damaged tissue (Medzhitov 2008). Inflammatory stimuli include a variety of factors such as microbial infections, necrotic tissue injuries, and cytokines (Takeuchi and Akira 2010). In terms of tissue homeostasis, inflammation should be resolved in a timely manner and damaged tissues should restore their normal structural and functional state. If inflammatory responses is not resolved, persistent inflammation may become a key factor causing chronic inflammatory diseases such as cancer, diabetes, sepsis, and atherosclerosis (Nathan and Ding 2010).

The innate immune system mediates inflammatory responses induced by microbial infection or tissue damage (Akira et al. 2006; Beutler et al. 2006; Takeuchi and Akira 2010). The innate immune responses are initiated by pattern recognition receptors (PRRs) which recognize pathogen-associated molecular patterns (PAMP) or damage-associated molecular patterns (DAMPs) (Takeuchi and Akira 2010). Among PRRs, toll-like receptors (TLRs) recognize PAMPs and transmit signals to downstream proteins through adaptor MyD88-dependent or MyD88-independent pathways, resulting in the activation of NF-κB or IRF3 protein to increase expression of pro-inflammatory cytokines and interferon-β1 (O'Neill and Bowie 2007; Takeuchi and Akira 2010).

Therefore, to maintain tissue homeostasis, TLR activation causing the inflammatory responses must be regulated, and aberrant regulation of TLR signaling is known to induce hyper-inflammatory diseases (Cook et al. 2004). Interestingly, anti-inflammatory activities of TGF-β cytokine have been reported in knockout mice studies, but the molecular mechanisms as to how TGF-β signaling is interconnected with innate immune responses and inflammations have not been addressed as much as other mechanisms mediated by TGF-β such as cell growth and apoptosis. Therefore, we review recent progresses on the TGF-β signaling pathway regulating inflammation and innate immune responses.

12.2 TGF-β and Inflammation

12.2.1 Effects of TGF-β Signaling Components on Inflammation

Previous studies on animal models of TGF-β signaling deficiency have clearly substantiated the importance of TGF-β signaling for immune functions and inflammation. The TGF-β1-null mutation in mice triggered an autoimmune inflammatory condition involving nuclear autoantibodies and autoreactive T cells. These mice had excessive inflammatory responses and developed a multifocal inflammatory disease resulting in cardiopulmonary complications that were ultimately lethal (Kulkarni et al. 1995; Shull et al. 1992). Histopathological analysis of these mice revealed massive infiltration of lymphocytes and macrophages in many organs, especially in the heart and lungs. In the spleen and the lymph nodes, inflammatory lesions included proliferation of immunoblasts and lymphoblasts in B and T cell zones. Inflammatory lesions were also seen in the pancreas, salivary glands, colon, and stomach of some of the TGF-β1(−/−) mice. In addition, conditional KO mice of TβRII gene, when it was disrupted by poly I:C induction in hematopoietic cells, showed a lethal inflammatory disease affecting multiple organs (Leveen et al. 2002). Bone marrow from conditional knockout mice of TβRII transferred to normal recipient mice caused a similar lethal inflammation, regardless of whether induction of TGF-β receptor deficiency occurred in donor animals before or in recipient animals

after transplantation. These results demonstrate that TGF-β signaling deficiency within cells of hematopoietic origin is sufficient enough to cause a lethal inflammatory disorder in mice. Another kidney-specific conditional KO mice of TβRII gene enhanced NF-κB signaling and renal inflammation including IL-1β and TNF-α in the model of unilateral urethral obstructive (UOO) nephropathy (Meng et al. 2012a). The important role of TGF-β in immune homeostasis has been previously suggested by the observation of multifocal immune-mediated inflammation in CD4-dnTGF-βRII transgenic mice similar to that of TGF-β1 KO mice. The CD4-dnTGF-βRII transgenic mice developed autoimmune disease characterized by inflammatory infiltration in several organs and the presence of circulating autoimmune antibodies, indicating that T cell homeostasis and prevention of inflammatory infiltration require TGF-β signaling in T cells (Gorelik and Flavell 2000). Among Smad proteins, Smad3 KO mice displayed impaired mucosal immunity and reduced T cell responsiveness (Ashcroft et al. 1999; Yang et al. 1999). Smad3-deficient mice were normal during embryonic and early postnatal development. After weaning, Smad3 KO mice invariably developed an illness associated with progressive leukocytosis, periodontitis, gastritis, colitis, and chronic infection with abscess formation adjacent to mucosal surfaces, suggesting that Smad3 has an important role in TGF-β-mediated regulation of T cell activation and mucosal immunity. The important role of Smad4 in inflammation has recently been suggested in a mouse model of unilateral urethral obstruction using conditional Smad4 knockout mice and in isolated Smad4 mutant macrophages and fibroblasts (Meng et al. 2012b). Disruption of Smad4 significantly enhanced renal inflammation as evidenced by a greater CD45(+) leukocyte and F4/80(+) macrophage infiltration and upregulation of IL-1β, TNF-α, MCP-1, and ICAM-1 in the obstructed kidney and in IL-1β-stimulated macrophages. These results suggest that Smad4 may also be a key regulator for the diverse roles of TGF-β1 in inflammation. The studies of animal models described in this review demonstrate the significant role of TGF-β in the regulation of T cell homeostasis and prevention of immune inflammation in many organs. These mice provide good models to study the pathogenic mechanisms in animal deficient of TGF-β signaling and to elucidate the specific cellular and molecular mechanisms of TGF-β that maintain homeostasis within the immune system.

Evidence of the anti-inflammatory role of TGF-β in human was shown in the study using biopsy specimens. Blockade of TGF-β in normal intestinal biopsies grown ex vivo and lamina propria mononuclear cells (LPMCs) downregulated T cell apoptosis and induced a significant increase in pro-inflammatory cytokines, including IFN-γ, TNF-α, IL-12, IL-6, IL-18, and IL-17, supporting an anti-inflammatory role of TGF-β in dampening T cell-mediated tissue-damaging responses in the human gut (Di Sabatino et al. 2008). On the contrary, the opposite results were observed in colonic biopsies of patients with ulcerative colitis (UC) or Crohn's disease (CD) (Babyatsky et al. 1996). The area of active inflammation in UC or CD showed an increased level of TGF-β expression. Surprisingly, the increased level of TGF-β in UC or CD did not exert an anti-inflammatory function (Monteleone et al. 2001). The reason was due to the overexpression of Smad7, an inhibitory Smad, in intestinal mucosa derived from active CD and most UC patients

(Monteleone et al. 2001). The overexpressed Smad7 blocked Smad3 activation through interfering with TGF-β type I receptor in these inflammatory diseases. Even though a number of studies are still performed to understand biological functions of TGF-β, these results basically suggest that in vivo functions of TGF-β signaling pathway are critically connected into immunological responses including innate immunity and adaptive immunity.

12.2.2 Anti-Inflammatory Role of TGF-β Signaling Pathway Suppressing Innate Immune Responses

TLRs have been known to be an important defense system responsible for invading pathogens. However, the molecular mechanism behind how their signaling cascades are regulated by anti-inflammatory cytokine TGF-β has recently been demonstrated in several reports. The initial report about an anti-inflammatory role of TGF-β suppressing TLR4 signaling pathway stated that TGF-β inhibited MyD88-dependent TLR4 signaling through decreasing the MyD88 protein levels in dose- and time-dependent manner (Naiki et al. 2005). However, the molecular mechanism behind the polyubiquitination and subsequent degradation of MyD88 by TGF-β was not clearly demonstrated in this report. Recently, the inhibitory Smad6 protein was shown to be responsible for the polyubiquitination and selective degradation of MyD88 through the recruitment of E3 ubiquitin ligases Smurf proteins (Fig. 12.1a) (Lee et al. 2011). These observations suggest that MyD88-dependent TLR signaling pathways such as TLR4 and TLR2 are negatively regulated by the Smad6-Smurf pathway when treated with TGF-β. The more interesting finding was that another inhibitory Smad, Smad7, is not involved in MyD88 degradation, indicating that the two inhibitory Smads, Smad6 and Smad7, have distinct roles as anti-inflammatory mediators (Lee et al. 2011). However, Smad6 is likely to be targeting another adaptor protein Pellino-1 when suppressing TLR4 signaling (Choi et al. 2006). TGF-β-induced Smad6 protein binds to Pellino-1, an adaptor protein binding to IRAK1 (Grosshans et al. 1999; Jiang et al. 2003; Moynagh 2009) and in turn disrupts IRAK1-mediated signaling complex in TLR4 signaling (Fig. 12.1a). The disruption of IRAK1-mediated signaling complexes through sequestering Pellino-1 by Smad6 resulted in the inhibition of NF-κB-mediated pro-inflammatory gene expressions (Choi et al. 2006). These findings strongly suggest that Smad6 is a pivotal component of TGF-β-mediated anti-inflammatory network and targets MyD88-dependent and Pellino-1-mediated TLR signaling. Interestingly, recent reports showed that Pellino-1 has additional binding partners in TLR signaling as well as IRAK1. Pellino-1 binds to TRIF-associated RIP1 protein (Chang et al. 2009). Another report indicated that Pellino-1 interacted with the IKKε/TBK1 complex which phosphorylates IRF3 (Smith et al. 2011). These findings suggest that TGF-β-induced Smad6 disrupts the RIP1- and IKKε/TBK1-mediated signaling complexes and subsequently inhibits MyD88-independent TLR3 signaling pathway.

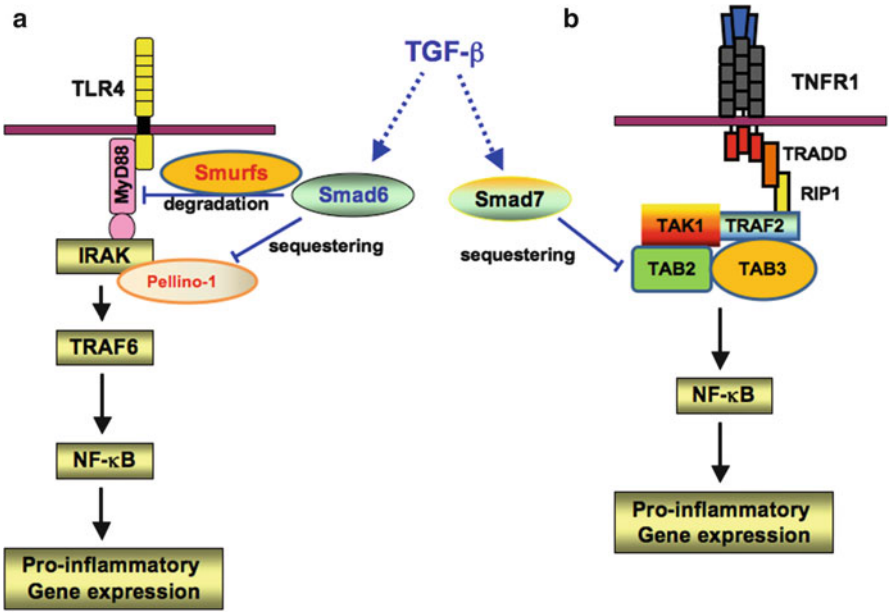

Fig. 12.1 Roles of the inhibitory Smads as mediators of anti-inflammatory TGF-β signaling. (**a**) TGF-β-induced Smad6 selectively degrades MyD88 protein in TLR4 signaling through recruiting E3 ubiquitin ligase Smurf proteins and also disrupts IRAK1-mediated signaling complex through sequestering the Pellino-1 protein. (**b**) TGF-β-induced Smad7 protein binds to TAB2 and TAB3 and subsequently suppresses TNF-α signaling through blocking the recruitment of the kinase TAK1 to the TRAF2 protein

In the case of Smad7, this molecule has been demonstrated to block pro-inflammatory TNF-α signaling (Hong et al. 2007). TGF-β-induced Smad7 interacts with the adaptors TAB2 and TAB3, and this interaction leads to the inhibition of TNF-α-induced NF-κB activity by blocking TAK1-associating proteins, TAB2 and TAB3, from forming a complex with TRAF2 (Fig. 12.1b) (Hong et al. 2007). Furthermore, Smad7 transgene expression in mouse skin under control of the keratin 5 promoter (K5-Smad7 mice) markedly suppressed inflammation and NF-κB nuclear translocation (Hong et al. 2007), suggesting that Smad7 is a critical mediator of the TGF-β pathway that blocks pro-inflammatory TNF-α signaling. Interestingly, Smad6 was not involved in TGF-β-mediated suppression of TNF-α signaling. TGF-β did not inhibit TNF-α-induced IL-6 expression in Smad7 knockdown primary peritoneal macrophages, whereas TGF-β still inhibited TNF-α-induced IL-6 expression in Smad6 knockdown primary peritoneal macrophages (Hong et al. 2007). Smad6 did not interact with TAB2. The Smad7 amino acids that are critical for TAB2-/TAB3-Smad7 interaction are not well conserved in Smad6. These findings strongly imply that Smad6 and Smad7 are differentially responsible for mediating anti-inflammatory TGF-β activity.

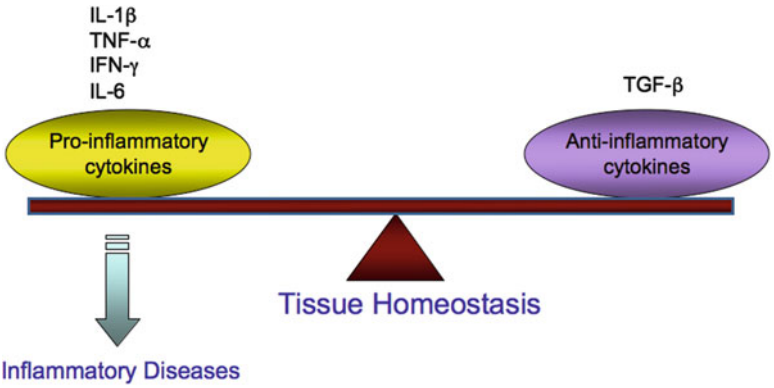

Fig. 12.2 Homeostatic balance between pro-inflammatory signals and anti-inflammatory TGF-β signaling under normal physiological conditions. The disruption of homeostatic balance between pro-inflammatory and anti-inflammatory signals, caused by excessive pro-inflammatory signals or abnormal reduction of anti-inflammatory signals, may be an important reason leading to the inflammatory diseases

Based on these findings, it is obvious that Smad6 and Smad7 play important roles in suppressing TLR4 signaling and pro-inflammatory TNF-α signaling, respectively. Although the TGF-β signaling pathway has been extensively studied, the physiological role of Smad7 and Smad6 requires further studies. The generation of Smad7 conditional knockout mice has been recently reported (Tojo et al. 2012); however, cell type-specific Smad6 conditional knockout mice are still unavailable. In vivo studies of these mice will elucidate how these molecules mediate TGF-β anti-inflammatory signaling.

12.2.3 The Inhibitory Smads as Cross Talkers of Intracellular Signaling Pathways

Recent reports suggest that inhibitory Smads may act as adaptors to facilitate cross talk between TGF-β signaling and pro-inflammatory signaling pathways. In this regard, Smad7 was extensively studied in the field of reciprocal regulation of TGF-β signaling and IFN-γ signaling. The pleiotropic cytokine IFN-γ suppressed TGF-β signaling through the increased expression of Smad7 by the activation of JAK-STAT1 pathway, which prevents the interaction of Smad3 with the TGF-β receptor and downregulates TGF-β signaling (Ulloa et al. 1999). Other groups reported another mechanism of IFN-γ-mediated suppression of TGF-β signaling (Ghosh et al. 2001). IFN-γ inhibited TGF-β signaling pathway through the sequestration of nuclear coactivator p300/CBP, which has been known to be associated with R-Smads (Ghosh et al. 2001). These results imply that the suppression of TGF-β signaling by IFN-γ will amplify the pro-inflammatory activity of IFN-γ under normal physiological conditions (Fig. 12.2).

In contrast, it has been reported that TGF-β inhibited IFN-γ-induced STAT1-dependent gene expression by enhancing STAT1-PIAS (a protein inhibitor of activated STAT1) interactions (Reardon and McKay 2007). However, these effects were shown in epithelial cells but not macrophages. Another group has shown that TGF-β inhibited IFN-γ-induced nitric oxide production in macrophages through the interaction of TGF-β type I receptor with IFN-γ receptor 1(IFNGR1) (Takaki et al. 2006). These results indicate that TGF-β suppresses the pro-inflammatory signal of IFN-γ to maximize its anti-inflammatory activity. Therefore, the anti-inflammatory and pro-inflammatory signals should be critically regulated to maintain tissue homeostasis (Fig. 12.2).

In addition, it was reported that TGF-β signaling is suppressed by NF-κB/RelA-dependent pathways (Bitzer et al. 2000). Activation of NF-κB/RelA by a variety of pathogenic and pro-inflammatory stimuli (TNF-α and IL-1β) increased transcription of the *Smad7* gene. This effect promoted the association of inhibitory Smad7 and type I TGF-β receptor upon TGF-β stimulation, leading to the suppression of TGF-β/Smad signaling. This gives further evidence that pro-inflammatory signals counteract anti-inflammatory signaling thresholds for TGF-β, dependent on physiological requirements to potentiate inflammatory responses. Similarly, a recent study supported the importance of a balance mechanism between TGF-β anti-inflammatory signal and pro-inflammatory signals. In this study, IL-1β or lipopolysaccharide (LPS) suppressed TGF-β-induced anti-inflammatory signaling in a NF-κB-independent manner through the interaction of TRAF6 with TGF-β type III receptor upon TGF-β stimulation, which then leads to pro-inflammatory factor-mediated attenuation of Smad2/3 phosphorylation (Lim et al. 2012). Although the antagonistic effects of IL-1β/LPS on TGF-β1 signaling may occur at multiple molecular levels, a fine modulation of the TGF-β receptors may critically affect cell fates by changing the kinetics of Smad2/3 phosphorylation. By this mechanism, IL-1β may potentiate inflammatory responses.

However, a recent report indicates that TRAF6-mediated polyubiquitination of transforming growth factor-β-associated kinase 1 (TAK1) activates NF-κB in HepG2 cells upon TGF-β1 treatment independent of p38 MAP kinase (Hamidi et al. 2012). This finding makes it difficult to understand the role of TGF-β1 in inflammatory responses. TAK1 is an important kinase in TGF-β noncanonical pathway, and TGF-β-induced Lys-63-linked polyubiquitination of TAK1 by TRAF6 is crucial for its activation and subsequent activation of p38 MAP kinase (Sorrentino et al. 2008). In this report, inflammatory stimuli, including IL-1β, TNF-α, and LPS, are able to induce TAK1 polyubiquitination and subsequent NF-κB activation in prostate cancer cells and RAW264.7 macrophages (Hamidi et al. 2012), suggesting that the increase of pro-inflammatory cytokines such as IL-6 may contribute to the formation of a tumor microenvironment. The interesting thing in this report is that TGF-β 1 also induces the same polyubiquitination of TAK1 and subsequent activation of NF-κB (Hamidi et al. 2012), suggesting that this phenomena may activate NF-κB survival signal in tumorigenesis. However, the authors did not show production of the pro-inflammatory cytokines in HepG2 cells upon TGF-β1 treatment as well as whether the activation of NF-κB upon TGF-β1 treatment is consistently observed in immune cells.

If TGF-β1-induced TAK1 and NF-κB activations are detected in immune cells such as macrophages and dendritic cells (DC), TGF-β1 is likely to have dual activities in inflammatory responses, as inflammatory or anti-inflammatory mediator and its activity might be differentially regulated according to cellular context or the related microenvironment.

On the other hand, the induction of Smad6 expression by pro-inflammatory signals is not reported, although Smad6 is transcriptionally induced by the anti-inflammatory cytokine TGF-β and responsible for TGF-β-mediated anti-inflammatory process (Afrakhte et al. 1998; Choi et al. 2006; Lee et al. 2011). A recent report showed that bacterial endotoxin LPS inhibits TGF-β-stimulated Smad6 expression in RAW264.7 macrophage cells (Kim and Kim 2011). The inhibition of TGF-β-induced Smad6 expression by LPS was mediated by phoshorylation of the Smad3 linker region through a TLR4-IRAK1-ERK1/2 pathway. The induction of Smad6 gene expression by pro-inflammatory signals has not been reported; however, we cannot exclude this possibility. It will be worth investigating whether pro-inflammatory signals potentiate inflammatory responses by stimulating Smad6 expression, which may counteract anti-inflammatory signaling thresholds for TGF-β/BMP.

12.2.4 A Role of TGF-β in T Cell Biology

TGF-β1 is one of the regulatory cytokines with pivotal functions in the control of inflammation. TGF-β1 directly targets T cells to ensure immune tolerance to self- and environmental antigens. Studies using animal models such as transgenic mice-expressing dominant-negative mutants of TβRII gene and T cell-specific TβRII KO mice indicated a principle role of TGF-β in T cell biology (Gorelik and Flavell 2000; Li et al. 2006; Lucas et al. 2000; Marie et al. 2006; Li and Flavell 2008). Generally, $CD4^+$ effector (helper) T cells are divided into two subsets, T helper type 1 (Th1) and T helper type 2 (Th2) cells, depending on the functions and cytokine production patterns. Th1 cells are characterized by their production of IFN-γ, a potent activator of cell-mediated immunity and thus responsible for removal of intracellular pathogens. Th2 cells that enhance eradication of parasitic infection are characterized by production of IL-4, IL-5, and IL-13. These helper T cells are critically regulated by regulatory T cells (Treg). Another subset of T helper cells is called Th17 cells which produce IL-17, a potent pro-inflammatory cytokine.

Recent studies have defined TGF-β as a critical regulator of thymic T cell development as well as a crucial player in peripheral T cell homeostasis, tolerance to self antigens, and T cell differentiation during the immune response (Li and Flavell 2008). Certain reports indicated that TGF-β has been implicated in the development of thymus-derived naturally occurring Treg cells (nTreg) (Liu et al. 2008). In addition, TGF-β promoted the differentiation of induced Treg (iTreg) cells from naïve T cells in peripheral tissues, which is enhanced by retinoic acid and IL-2 (Fig. 12.3) (Benson et al. 2007; Coombes et al. 2007; Davidson et al. 2007; Mucida et al. 2007;

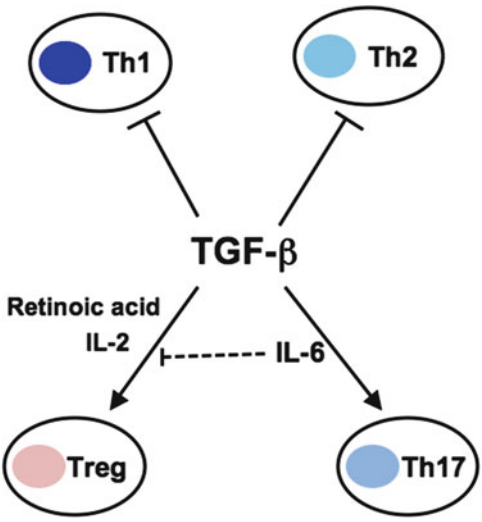

Fig. 12.3 A role of TGF-β in T cell differentiation and tolerance. Active TGF-β directly inhibits the differentiation of Th1 and Th2 cells. TGF-β promotes the generation of the Th17 cells from naïve T cells in the presence of IL-6 and the differentiation of naïve T cells into iTreg cells is simultaneously inhibited

Zheng et al. 2007). It has been known that TGF-β induces the expression of Foxp3, a master regulator of Treg cells (Chen et al. 2003). Foxp3 expression in Treg cells inhibited secretion of pro-inflammatory cytokines, including IL-2, IFN-γ, IL-4, and IL-17. Foxp3 expression also enhanced expression of anti-inflammatory cytokines, IL-10 and TGF-β, and increased an inhibitor for co-stimulation, CTLA4 (Bettelli et al. 2005; Fontenot et al. 2003; Zhou et al. 2008), eventually controlling and dampening inflammation as well as promoting tolerance. In particular, the finding that TGF-β in breast milk induces tolerance through $CD4^+$ Treg cells strongly supports the importance of TGF-β-Treg axis in the immune system (Verhasselt et al. 2008). That is, airborne antigens were efficiently transferred from the mother to the neonate through breast milk and breastfeeding-induced tolerance during lactation was mediated by $CD4^+$ Treg cells depending on the presence of TGF-β (Verhasselt et al. 2008). In addition, it has been reported that TGF-β administrated through the oral route retains sufficient biological activity in the intestinal mucosa and enhances the induction of oral tolerance (Ando et al. 2007). These studies collectively emphasize the crucial role that TGF-β plays to control the immune responses, such as tolerance induction.

While the differentiation of iTreg cells by TGF-β is inhibited in the presence of pro-inflammatory cytokine IL-6, TGF-β promotes the generation of the Th17 cells from naïve T cells in the presence of IL-6 (Fig. 12.3) (Bettelli et al. 2006). When naïve T cells differentiated into Th17 cells upon TGF-β plus IL-6, the differentiation of naïve T cells into iTreg cells was simultaneously inhibited (Fig. 12.3). This differential regulation of naïve T cells by TGF-β implies that the two T cell lineages are closely interconnected showing a mutually exclusive pattern, and has important functions in immune responses. The mechanisms underlying the regulation of Th17 and iTreg cell differentiation remains to be understood.

Alternatively, another study showed that dendritic cells (DCs) are involved in T cell responses regulated by TGF-β. Upon stimulation of naïve T cells by DCs in the presence of TGF-β, antigen-specific Foxp3$^+$ Treg cells were generated (Yamazaki et al. 2006). Treg cells secreted the latent form of TGF-β1 associated with the latency-associated protein (LAP) (Nakamura et al. 2001; Oida et al. 2003), although it is still controversial whether Treg cells are the primary source for TGF-β production. In turn, αvβ8 integrin, which was expressed on DCs, degraded LAP protein through interaction with LAP and released active TGF-β (Taylor 2009). Conditional deletion of integrin αvβ8 on DCs showing autoimmune diseases (Travis et al. 2007) strongly supported the importance of interaction between Treg and DC cells. Active TGF-β finally inhibited the differentiation of Th1 and Th2 cells from naïve T cells and promoted the differentiation to iTreg (Fig. 12.3). Furthermore, certain reports indicate that the mRNA of transmembrane protein called glycoprotein A repetitions predominant (GARP) is selectively induced by TCR stimulation in human Foxp3$^+$ Treg cells and the GARP protein acts as a receptor for latent TGF-β (Wang et al. 2008; Probst-Kepper et al. 2009; Stockis et al. 2009; Wang et al. 2009). These results imply that GARP is a highly specific marker which could be used for the identification of activated Treg cells, and the binding of TGF-β to GARP is an important clue for explaining the activation of Treg cells. In conclusion, a number of studies about the roles of TGF-β in T cells using in vivo or in vitro systems strongly emphasize that TGF-β's immunological function might be more important to understand the pathogenesis of human diseases.

12.3 Conclusion

Numerous studies about TGF-β's biological functions suggest that TGF-β exerts powerful anti-inflammatory activity and is the master controller of immune responses. Therefore, TGF-β-mediated signaling pathway should be critically controlled together with other essential intracellular signaling pathways to maintain homeostatic balance under normal physiological conditions. If this homeostatic balance is disrupted by unexpected stimuli or genetic mutations of TGF-β signaling-related genes, human diseases such as autoimmune and inflammatory diseases may develop. The typical examples are inflammatory bowel diseases such as Crohn's disease. Recently, many researchers are interested in autoimmune diseases and inflammatory diseases exerted by Th17 cells. In particular, Th17 cells and their effector molecules such as IL-17, IL-21, IL-22, and GM-CSF are known to be associated with the pathogenesis of rheumatoid arthritis, systematic lupus erythemaosus, multiple sclerosis, psoriasis, IBD, allergy, and asthma (Miossec et al. 2009; Wilke et al. 2011). Although the TGF-β/Smad signaling pathway is extensively studied in T cell differentiation, Treg cells, and Th17 cells, it has not yet been addressed as to how the inhibitory Smads, Smad6 and Smad7, act on the homeostasis of T cells. Therefore, it will be interesting to study whether inhibitory Smads mediate the role of TGF-β in T cell biology.

Drugs have been and are being developed to inhibit TGF-β activity, including receptor kinase antagonists, neutralizing antibodies, and antisense oligonucleotides. Since inhibitory Smads mediate some of TGF-β's anti-inflammatory activities, they may have a therapeutic potential for treatment of TLR4- or inflammation-related diseases. Therefore, if the detailed mechanisms of TGF-β's anti-inflammation activity are revealed in the future, it will be quite promising for drug development targeting or mimicking TGF-β or its signaling components.

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Part III
Cardiovascular Diseases

Chapter 13
TGF-β and Cardiovascular Disorders

Laurens A. van Meeteren, Marie-José Goumans, and Peter ten Dijke

Abstract Transforming growth factor β (TGF-β) is a secreted pleiotropic cytokine that is involved in a wide range of biological processes and has essential roles in development and tissue homeostasis. TGF-β elicits cellular effects by activating serine/threonine kinase receptors located at the plasma membrane and intracellular Smad effector proteins. TGF-β is an important (cardio)vascular regulator as shown by many in vitro studies on cultured endothelial and mural cells, in vivo studies in animal models, and genetic studies in which mutations in human TGF-β signaling components have been causally linked to cardiovascular diseases. Here we review recent progress in our understanding of the (dys)function of TGF-β in the cardiovascular system.

Keywords Angiogenesis • Cardiovascular • Endothelium • TGF-β

13.1 Introduction

Transforming growth factor β (TGF-β) is one of the most studied members of a large family of structurally related secreted pleiotropic cell to cell signaling molecules. Different members of the family include the activins and bone morphogenetic proteins (BMPs). Many of these cytokines have essential roles in numerous processes during development, but also in maintenance of tissue homeostasis and tissue repair in the adult (Massagué 1998). Not surprisingly,

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when their action is perturbed, they have an important role in pathological conditions, like vascular diseases, cancer, and fibrosis (Blobe et al. 2000; ten Dijke and Arthur 2007).

The ligands of the TGF-β family mediate their effects by binding specific single transmembrane receptors at the cell membrane (Feng and Derynck 2005). Upon ligand-induced heteromeric complex formation, the type I receptor is phosphorylated by the type II receptor. There are seven different type I receptors (ALK1 to ALK7) (also known as activin receptor-like kinases (ALK)) and five type II receptors (activin receptor type IIA (ActRIIA), activin receptor type IIB (ActRIIB), BMP type II receptor (BMPRII), TGF-β type II receptor (TGF-βRII), and AMH type II receptor (AMHRII)). Different ligands can bind different combinations of type I and type II receptor thereby creating specificity of signaling. TGF-β signals mostly via ALK5 and TGF-βRII, activins via ALK4 with ActRIIA or ActRIIB, and BMPs signal via ALK1, 2, 3, and 6 together with BMPRII, ActRIIA, or ActRIIB. For regulation of endothelial function by TGF-β, ALK1 and ALK5 signaling are most important (van Meeteren and ten Dijke 2012).

After binding of the ligand the type I receptor phosphorylates specific transcription factors, receptor regulated (R)-Smads. Upon activation by type I receptors, R-Smads form heteromeric complexes with the common mediator (Co)-Smad (Smad4) and these heteromeric complexes accumulate into the nucleus, where they regulate the transcription of specific target genes (Moustakas and Heldin 2009).

Inhibitory Smads (I-Smads) inhibit the activation of R-Smads by competing for type I receptor interaction and by recruiting phosphatases and ubiquitin ligases to the activated receptor complex leading to dephosphorylation or proteosomal degradation of the receptor complex (Itoh and ten Dijke 2007). R-Smads can be divided into two groups based on the type I receptor that is activating them. The first group consists of Smad1, 5, and 8 and these are activated by ALK1, 2, 3, and 6. The second group contains Smad2 and 3 and is activated by ALK4, 5, and 7. In addition to Smad signaling, TGF-β and BMP signaling can result in activation of pathways where Smads are not directly involved. Non-Smad pathways include various branches of MAP kinase pathways, Rho-like GTPase signaling pathways, and PI3K/AKT pathways (Moustakas and Heldin 2005; Zhang 2009). Non-Smad signaling pathway have been found to modulate Smad signaling and vice versa (Moustakas and Heldin 2005) (Figs. 13.1).

Co-receptors are receptors that do not signal by themselves since they lack intracellular enzymatic motifs such as kinase domains (Kirkbride et al. 2005). For TGF-β signaling co-receptors endoglin and betaglycan (also called TGF-β receptor III) have been identified (Cheifetz et al. 1992; Wang et al. 1991). Both receptors are structurally related and have a small intracellular tail, a single transmembrane domain, and a large extracellular domain. Endoglin is highly expressed in proliferating endothelial cells, hence its name endoglin.

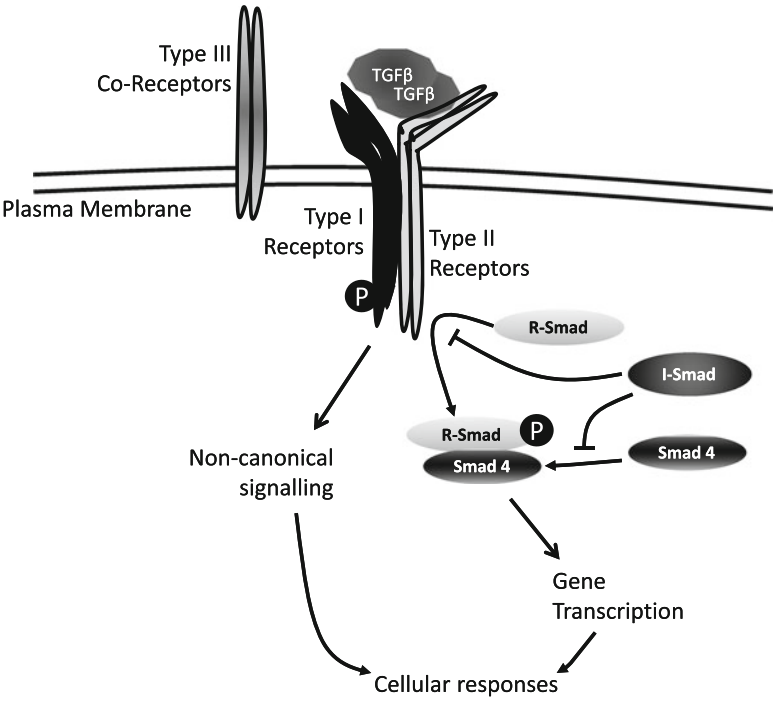

Fig. 13.1 Basic signaling unit of the TGF-β system. TGF-β family ligands such as TGF-β and BMPs bind a complex of type I receptors, type II receptors, and possibly a type III co-receptor leading to activation of R-Smads. R-Smads then form a complex with Smad4 leading to specific gene-transcription. I-Smads inhibit the activation of R-Smads and complex formation of Smad4 with R-Smads. In addition, the receptor complex can also activate non-Smad pathways

13.2 TGF-β Signaling in Vascular Biology

13.2.1 Regulating Endothelial Function by TGF-β

Genetic studies in mice and humans revealed the importance of components of the TGF-β signaling pathway in vascular morphogenesis and angiogenesis. Deletion of components of the TGF-β pathway (ligands, receptors, and intracellular Smad mediators) in mice leads to embryonic lethality due to vascular abnormalities (see below) (van Meeteren et al. 2011).

To regulate the activation state of endothelial cells TGF-β can differentially activate two type I receptors, ALK1 and ALK5 (Goumans et al. 2002; Oh et al. 2000). ALK5 is expressed in most tissues, but ALK1 expression is restricted to endothelial cells. TGF-β-induced ALK5 signaling activates Smad2 and Smad3 phosphorylation resulting in inhibition of endothelial cell proliferation, migration, and organization

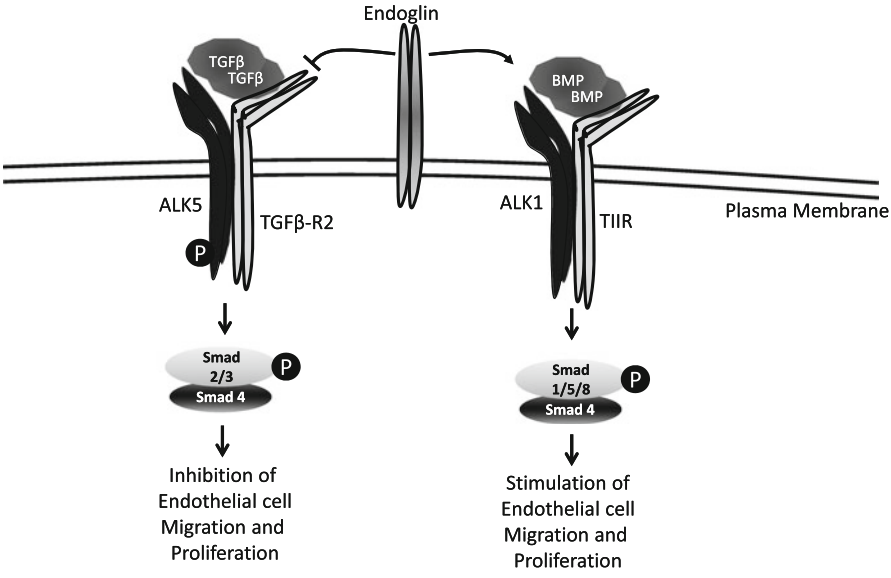

Fig. 13.2 TGF-β signaling in endothelial cells. BMP binding to ALK1 (in complex with a type II receptor and endoglin) activates Smad1, 5, and 8 leading to stimulation of endothelial cell migration and proliferation. ALK1 is antagonized by ALK5 that is activated by TGF-β leading to Smad2 and 3 activation and endothelial cell quiescence

(Goumans et al. 2003) (Fig. 13.2). The ALK5 kinase inhibitor SB-431542 enabled proliferation and sheet formation of embryonic stem cell-derived endothelial cells. SB-431542 upregulated the expression of claudin-5, an endothelial-specific component of tight junctions, suggesting a role of ALK5 signaling in regulating vascular permeability (Watabe et al. 2003). In addition, VEGF and inhibitors of TGF-β type I receptor kinase synergistically promoted blood vessel formation by inducing β 5-integrin expression (Liu et al. 2009). In contradiction to these findings, in vivo, SB-431542 has been reported to induce enhanced delivery of nanoparticles to the tumor tissue extravascular space due to increased vessel leakiness (Kano et al. 2007). Indeed, ALK5 has been reported before to be important for TGF-β-induced permeability and cytoskeletal remodeling of endothelial cells (Birukova et al. 2005). Interestingly a major component of endothelial cell–cell junctions, VE-cadherin, was found to interact with ALK5, ALK1, endoglin, and TGF-βRII, which possibly reveals the link between permeability and TGF-β signaling (Rudini et al. 2008). In conclusion, ALK5 signaling results in keeping endothelial cells in a quiescent state.

TGF-β-induced ALK1 signaling activates Smad1 and Smad5 leading to endothelial cell proliferation, migration, and organization (Goumans et al. 2003). A central intracellular effector of ALK1 is Id1; its upregulation was shown to be required for TGF-β/ALK1-induced endothelial cell migration and tube formation (Valdimarsdottir et al. 2002) and inhibition of ALK1 was shown to inhibit endothelial cell sprouting (van Meeteren et al. 2012). However, inhibitory effects of ALK1

signaling on endothelial cells have also been reported (David et al. 2007; Lamouille et al. 2002; Mallet et al. 2006). The effect of ALK1 is likely dependent on cellular context.

Although ALK1 and ALK5 have opposing effects on endothelial cells, they do interact with each other physically. ALK5-deficient endothelial cells are not only defective in ALK5 signaling but also show impaired ALK1 responses; ALK5 was found to be essential for recruitment of ALK1 into a TGF-β receptor complex, and the kinase activity of ALK5 is essential for full ALK1 activation (Goumans et al. 2003). In the presence of fibronectin ALK1 activity is not dependent on ALK5 activity since in the presence of fibronectin an ALK5 inhibitor has no effect on ALK1-mediated Smad1/5/8 phosphorylation (Tian et al. 2012). On the other hand, ALK1 can directly antagonize ALK5 signaling at the level of Smads (Goumans et al. 2002; Oh et al. 2000). Taken together, the cross talk between ALK1 and ALK5 signaling provides endothelial cells with a sophisticated mechanism to fine-tune endothelial function.

Endothelial cells can express both the TGF-β co-receptors endoglin and betaglycan. Endoglin positive but betaglycan negative endothelial cells are responsive to TGF-β-1 and -3, but not -2 (Cheifetz et al. 1990). In endothelial cells that express both co-receptors endoglin can form a complex with betaglycan and the TGF-β receptor complex simultaneously (Wong et al. 2000).

Endoglin is predominantly expressed in highly proliferating endothelial cells, but is also expressed in hematopoietic cells, syncytiotrophoblasts of term placenta, stromal cells, smooth muscle cells, and mesenchymal cells (Bot et al. 2009; Dağdeviren et al. 1998). Ectopic expression of endoglin inhibits TGF-β-induced growth inhibition of endothelial cells, monocytes and myoblasts (Lastres 1996; Lebrin et al. 2004), and extracellular matrix deposition (Obreo et al. 2004). Endoglin plays an important role in balancing ALK1 and ALK5 signaling pathways. Endoglin overexpression potentiates TGF-β/ALK1 signaling and inhibits TGF-β/ALK5 signaling, whereas endoglin knockdown resulted in impaired TGF-β/ALK1 signaling responses (Blanco et al. 2005; Lebrin et al. 2004). Endothelial cells isolated from endoglin heterozygote or homozygote embryos displayed significantly reduced proliferation and migration, increased collagen production, and decreased nitric oxide (NO) synthase expression and VEGF secretion (Jerkic et al. 2006). Hypoxia upregulates endoglin levels and this upregulation was shown to protect endothelial cells from hypoxia-induced apoptosis (Li et al. 2003).

A general feature of co-receptors is that they can be cleaved from the cell membrane with the consequence that they exist as soluble forms. Indeed, also betaglycan and endoglin can be shedded by membrane type matrix metalloprotease-1 (MT-MMP) from the plasma membrane giving rise to a soluble betaglycan and soluble endoglin (sol-endoglin) (Hawinkels et al. 2010; Velasco-Loyden et al. 2004). Interestingly, sol-endoglin levels in plasma are a prognostic marker for several pathological conditions, including pregnant women suffering from preeclampsia (Levine et al. 2006; Liu et al. 2012). Furthermore, several labs have reported increased levels of sol-endoglin in serum from cancer patients as a marker of poor prognosis (Li et al. 2000a).

13.2.2 Regulating Mural Cell Function by TGF-β

Both endothelial cells and their supporting cells, such as vascular smooth muscle cells (VSMCs) and pericytes, are needed for proper endothelial function. Tight regulation and close coordination between endothelial cells and VSMCs and pericytes are needed to form a mature vascular network (Armulik et al. 2005, 2011). VSMCs express type I and type II receptors for TGF-β family members (Bobik 2006), and TGF-β is a potent stimulator of VSMC differentiation (Owens 1995). VSMCs form gap junctions with endothelial cells and upon receiving signals from VSMCs, endothelial cells line up and recruit more VSMCs. TGF-β is an important regulator of the interaction between endothelial cells and the supporting cells. Endothelial cells produce latent TGF-β that upon endothelial–VSMC interaction can be activated leading to VSMC differentiation and function (Ding et al. 2004). Knockout mice of several TGF-β signaling players reveal the significance of TGF-β signaling in VSMC–endothelial cell interaction. ALK1- and endoglin knockout mice show defective remodeling of the primary capillary plexus of the yolk sac and failure in SMC development (Arthur et al. 2000; Goumans and Mummery 2000; Oh et al. 2000). TGF-βRII, Smad5, Smad1, and TGF-β1 knockout mice show defects in vasculature structure or blood vessel organization indicative of a defect in endothelial cell lining and impaired VSMC development (ten Dijke and Arthur 2007).

13.2.3 Lessons from Knockout Mice

TGF-β ligands receptors and downstream signaling components are involved in various aspects of vascular biology, in physiology as well as pathophysiology and in development as well as the adult stage (ten Dijke et al. 2008).

Knockout studies in mice offered the first indication that TGF-β signaling plays an essential role in vascular function and development. We will focus mainly on the animal models with disrupted TGF-β signaling components that exhibit defects in angiogenesis and vasculogenesis. For a summary of these knockouts, see Table 13.1.

The first reports on TGF-β knockout mice showed that TGF-β was only necessary for postnatal survival and not for embryonic development (Kulkarni et al. 1993; Shull et al. 1992). However, subsequently it was reported that on the genetic background used for the first studies placental and lactational transfer of maternal TGF-β1 from heterozygous mothers to knockout embryos and pups could have rescued their embryonic lethality (Kallapur et al. 1999; Letterio et al. 1994). In a mixed 129×NIH/Ola×C57BL/6 background, 50 % of the mutant embryos die around embryonic day 10.5 (E10.5) due to inadequate yolk sac development (Bonyadi et al. 1997; Dickson et al. 1995) as a result of failure in both vasculogenesis and hematopoiesis. A defect in terminal differentiation of the endothelial cells in the yolk sac affecting endothelial tube formation and/or its integrity resulting in insufficient capillary tube formation and weak vessels was observed. However, there was no

Table 13.1 List of TGF-β signaling component knockout mice that show vascular defects

Gene	Phenotype knockout mice	References
Ligands		
TGF-β1	Embryonic lethal (E10.5) with vascular defects such as inadequate yolk sac development, defective vasculogenesis, and hematopoiesis	Dickson et al. (1995), Kulkarni et al. (1993), Letterio et al. (1994), Shull et al. (1992)
Receptors		
ALK1	Embryonic lethal (E11.5) due to severe vascular abnormalities such as large shunts between arteries and veins. Also defects in differentiation and recruitment of VSMCs	Oh et al. (2000), Urness et al. (2000)
ALK5	Embryonic lethal (E11.5), severe defects in vascular development of the yolk sac and placenta, and absence of circulating red blood cells	Larsson et al. (2001)
Co-receptors		
Endoglin	Embryonic lethal (E10.5) due to angiogenesis defects and defects in VSMC differentiation	Arthur et al. (2000), Bourdeau et al. (1999), Carvalho et al. (2004), Li (1999)
Betaglycan	Embryonic lethal (E13.5) with defects in coronary vessels and increased apoptosis in the liver	Compton et al. (2007), Stenvers et al. (2003)
Smads		
Smad4	Endothelial-specific knockout is embryonic lethal (E10.5) due to cardiovascular defects and defects in VSMC differentiation and recruitment	Lan et al. (2007)
Smad5	Embryonic lethal (E10.5) due to angiogenesis defects in the yolk sac and enlarged blood vessels that failed to recruit VSMCs	Yang et al. (1999)

obvious defect in vascular development within the TGF-β1-deficient embryos at E9.5. TGF-β2 and TGF-β3 knockout mice die perinatal with no obvious vascular defects (Kaartinen et al. 1995; Sanford et al. 1997).

Acvrl1 (encoding for mouse ALK1) homozygous knockout embryos die at midgestation (around E11.5) due to severe vascular abnormalities such as large shunts between arteries and veins and hyperdilation of large vessels. Furthermore, defects in differentiation and recruitment of supporting cells such as VSMCs were observed (Oh et al. 2000; Urness et al. 2000). Interestingly, a zebrafish with a mutation in ALK1 suffers from an abnormal circulation pattern in which blood flows through a limited number of dilated cranial vessels and failure to perfuse the trunk and tail (Roman et al. 2002).

Mice deficient for ALK5 die also at midgestation, exhibiting severe defects in vascular development of the yolk sac and placenta, and an absence of circulating red blood cells. Endothelial cells from ALK5-deficient embryos show enhanced cell proliferation, improper migratory behavior, and impaired fibronectin production in vitro, defects that are associated with the vascular defects seen in vivo (Larsson et al. 2001).

When *Alk5* was knocked out in only ALK1 expressing endothelial cells, no vascular phenotype was observed. This would suggest that ALK5 plays no important roles in endothelial cells (Park et al. 2008) in line with earlier reports from the same lab that found no detectable expression of ALK5 in endothelial cells (Seki et al. 2006). However, it is in conflict with earlier studies that showed that conditional *Alk5* crossed with the endothelial-specific *tie-1*-cre mice did show a lethal vascular phenotype (Carvalho et al. 2004). This discrepancy is most likely due to different expression pattern between the *tie-1*-cre and the *alk1*-cre. *Tie-1*-cre is supposedly expressed earlier in endothelial cells, and therefore it could mean that ALK5 plays an essential role in the earliest stages of mouse vascular development. Indeed, in the *alk1*-cre mice cre is only expressed at a stage when the *tie-1*-cre ALK5 embryos have already died.

Knockout mice for many other ALKs are also embryonic lethal but at stages before vascular development. For example knockout mice for *Alk2*, *Alk3*, and *Alk4* die around E7.5 (Goumans and Mummery 2000). Therefore it cannot be excluded that also these have roles in the development of the vascular system. By crossing conditional knockout mice for these receptors with an endothelial-specific inducer these roles could be revealed in the future.

Mice deficient for the ALK1 co-receptor endoglin were described by three independent groups. All endoglin knockout mice showed early embryonic lethality at E10.5–11.5 days caused by angiogenesis defects of the yolk sac and abnormal heart development (Arthur et al. 2000; Bourdeau et al. 1999; Li 1999). The formation of the primitive vascular plexus in the yolk sac occurred normally but the successive branching and remodeling associated with angiogenesis fails to proceed. Subsequent analysis of the endoglin knockout mice showed that endoglin is required for downstream TGF-β signaling from the endothelial cell to the adjacent smooth muscle cell to promote smooth muscle cell differentiation (Carvalho et al. 2004). Mice deficient for the co-receptor betaglycan die around E13.5 due to defects in the formation of the coronary vessels and increased apoptosis in the liver (Compton et al. 2007; Stenvers et al. 2003).

Smad1, *2*, and *4* deficient mice all die before vascular development starts due to defects and abnormalities in germ layer formation. In addition, the allantois in Smad1 knockouts fails to connect with the placenta (Huang et al. 2002).

Endothelial-specific *Smad4* knockouts die at E10.5 due to cardiovascular defects, including attenuated vessels sprouting and remodeling, collapsed dorsal aortas, enlarged hearts with reduced trabeculae, and failed endocardial cushion formation. Furthermore, vessels that formed showed impaired development of VSMCs and association between endothelial and VSMCs. Noticeably, *Smad4*-deficient endothelial cells from these mice demonstrated an intrinsic defect in tube formation in vitro (Lan et al. 2007).

Smad5 knockout mouse embryos die between days 10.5 and 11.5 due to defects in angiogenesis. The mutant yolk sacs lacked normal vasculature and had irregularly distributed blood cells, although they contained hematopoietic precursors capable of erythroid differentiation. In addition, *Smad5* mutant embryos had enlarged blood vessels surrounded by decreased numbers of VSMCs, suffered massive apoptosis of mesenchymal cells (Yang et al. 1999).

13.2.4 Endothelial-to-Mesenchymal Transition

Recent evidence has demonstrated that endothelial cells can have a remarkable plasticity. By a process called endothelial-to-mesenchymal transition (EndMT) endothelial cells convert to a more mesenchymal cell type that can give rise to cells such as fibroblasts, but also bone cells. EndMT is essential during embryonic development and tissue regeneration (van Meeteren and ten Dijke 2012). Interestingly, it also plays a role in pathological conditions like fibrosis of organs such as the heart and kidney (Goumans et al. 2008). In addition, EndMT contributes to the generation of cancer-associated fibroblasts that are known to influence the tumor-microenvironment favorable for the tumor cells. EndMT is a form of the more widely known and studied epithelial-to-mesenchymal transition (EMT) (Thiery et al. 2009).

Many endothelial cells can be induced to undergo EndMT when stimulated with TGF-β or Notch ligands (Frid et al. 2002; Ishisaki et al. 2003; Noseda et al. 2004; Timmerman et al. 2004; Zeisberg et al. 2007a, b), albeit that embryonic cells are more plastic than adult cells. The molecular mechanism behind TGF-β-induced EndMT has been found to involve the Snail family of transcription repressors. In mouse embryonic stem cell derived endothelial cells TGF-β stimulation induced EndMT and increased the expression of Snail. This upregulation of Snail by TGF-β was shown to be dependent on the activation of Smad, MEK, PI3K, and p38 MAPK by TGF-β (Medici et al. 2010). Subsequent knockdown of Snail blocked the TGF-β-induced EndMT (Kokudo et al. 2008). Although overexpression of Snail was sufficient to induce EMT (Cano et al. 2000), for EndMT Snail expression alone is insufficient. The inhibitor of Snail, GSK-3β, needs to be inhibited by phosphorylation by kinases such as AKT to induce EndMT (Medici et al. 2010).

Notch can, as TGF-β, induce EndMT in endothelial cells in vitro. In this Notch-induced EndMT the Snail family member Slug has been shown to be essential (Leong et al. 2007). Snail and Slug are known to repress the expression of VE-cadherin (Lopez et al. 2009). Since VE-cadherin is essential for endothelial cell–cell junctions, this obviously could provide a link to a mechanism by which EndMT occurs. A different factor involved in TGF-β-induced EndMT was shown to be plasminogen activator inhibitor-1 (PAI-1). Although elevated levels of PAI-1 are implicated in tissue fibrosis (Ghosh and Vaughan 2012), lack of PAI-1 in the heart is associated with the development of cardiac fibrosis in aged mice (Ghosh et al. 2010). It was shown that in the PAI-deficient endothelial cells of these mice both Smad and non-Smad TGF-β signaling is spontaneously activated. This spontaneous activation leads to EndMT and subsequently the fibrosis observed in these animals. In addition, it was recently shown that c-Abelson tyrosine kinase (c-Abl) and Protein Kinase C (PKC)-δ are crucial for TGF-β-induced EndMT, and therefore that imatinib mesylate and rottlerin (inhibitors of c-Abl and PKC-δ, respectively) might be effective therapeutic agents for fibroproliferative pathologies in which EndMT plays a role (Li and Jimenez 2011).

It has also been reported that pathways other than TGF-β can lead to EndMT. In myocardial infarction (MI) for example, canonical (β-catenin-dependent) Wnt signaling is induced 4 days after experimental MI in the subepicardial endothelial cells

and perivascular cells. Coincidently with canonical Wnt activation EndMT was also triggered after the infarction. In addition, canonical Wnt signaling induced mesenchymal characteristics in cultured endothelial cells, suggesting a direct role of canonical Wnt signaling in EndMT (Aisagbonhi et al. 2011).

13.3 TGF-β in Vascular Pathologies

Given the importance of TGF-β signaling for blood vessels, it is not surprising that TGF-β signaling is involved in several pathological conditions concerning the endothelium (Fig. 13.3).

13.3.1 Hereditary Hemorrhagic Telangiectasia

Hereditary hemorrhagic telangiectasia (HHT), or Osler–Rendu–Weber syndrome, is an autosomal dominant vascular dysplasia characterized by the development of mucocutaneous telangiectasias and arteriovenous malformations in the lungs, brain, liver, and gastrointestinal tract (Abdalla and Letarte 2006). Many HHT patients suffer from recurrent nosebleeds, which severely affect their quality of life and are clinically difficult to treat. Mutations in three different genes have been shown to be responsible for HHT. Interestingly these are *ENG* (encoding endoglin), *ACVRL1* (encoding ALK1), and *SMAD4*, and the proteins encoded by these genes are all important players in TGF-β signaling in endothelial cells. Mutations in *ENG* cause

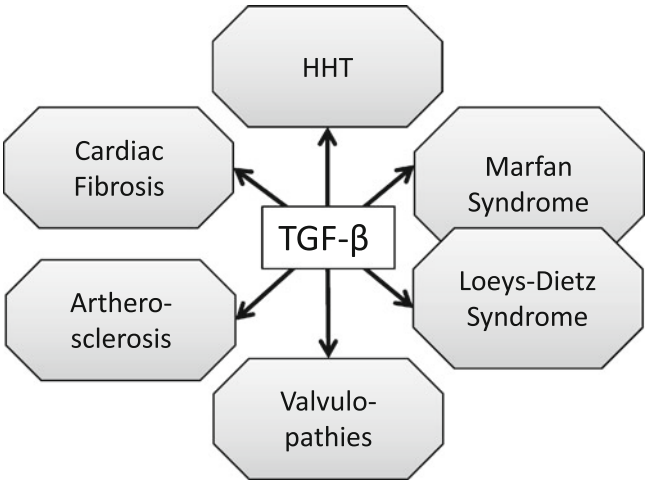

Fig. 13.3 TGF-β plays a central role in vascular pathologies like Marfan and Loeys–Dietz syndrome, hereditary hemorrhagic telangiectasia (HHT), atherosclerosis, cardiac fibrosis, and congenital heart valve defects

HHT type 1 (HHT-1) (McAllister et al. 1994); *ACVRL1* mutations cause HHT-2 (Johnson et al. 1996); and mutations in *SMAD4* cause a syndrome consisting of both juvenile polyposis and a HHT phenotype (Gallione et al. 2006). Endoglin heterozygous knockout mice have dilated and fragile blood vessels which resemble clinical manifestations of HHT-1 patients (Arthur et al. 2000; Bourdeau et al. 2001). Endothelial-specific ALK1 knockout mice suffer from vascular malformations mimicking all pathologic features of HHT-2 (Park et al. 2009). Several clinical trials in HHT patients are ongoing with anti-angiogenesis agents, including bevacizumab, a neutralizing antibody against VEGF and thalidomide to inhibit bleedings and other vascular malformations associated with HHT. Recently, it was reported that thalidomide treatment of a small group of HHT patients reduced the severity and frequency of nosebleeds (epistaxis) in the majority of the patients. In addition, in heterozygous endoglin knockout mice thalidomide treatment stimulated mural cell coverage and thus rescued vessel wall defects. Thalidomide treatment increased platelet-derived growth factor-B (PDGF-B) expression in endothelial cells and stimulated mural cell activation. The effects of thalidomide treatment were partially reversed by pharmacological or genetic interference with PDGF signaling from endothelial cells to pericytes (Lebrin et al. 2010).

13.3.2 Marfan Syndrome and Loeys Dietz Syndrome

Marfan syndrome (MFS) is a genetic disorder that has been linked to mutations in the *FBN1* gene, which encodes for the extracellular matrix glycoprotein Fibrillin-1 (Dietz et al. 1991). This protein forms an important component of elastic fibers in the aorta, ligaments, eye, and other tissues. Inactivating *FBN1* mutations in MFS affect cardiovascular, pulmonary, ocular, and skeletal tissues, among other tissues (Ramirez and Dietz 2007). Most dangerous are the aortic aneurysms, which carry the risk of sudden rupture and death. Initially it was thought that MFS was caused by structural defects in elastic fibers. However, fibrillin-1 not only serves an important role in providing structural integrity of connective tissues, but also acts as reservoir for growth factors such as TGF-β (ten Dijke and Arthur 2007). The latter function appears to be prominently associated with the vascular pathologies of MFS (Bolar et al. 2012). Fibrillin-1 contains multiple latent TGF-β binding motifs, and can associate with LTBP-1 and control the availability of active extracellular TGF-β (Chaudhry et al. 2007). Insufficient or nonfunctional fibrillin-1 as result of a *FBN1* mutation may lead to the release of bioactive TGF-β, and thereby trigger elevated TGF-β/Smad2 signaling (Habashi et al. 2006; Neptune et al. 2003).

A disorder closely related to MFS is Loeys Dietz syndrome (LDS). The latter has been linked to mutations in *ALK5*, *TGFBR2*, and *TGF-β2* (Lindsay et al. 2012; Loeys et al. 2005). LSD overlaps clinically to a large extent with MFS, but LSD has also unique properties, including widely spaced eyes. Some of the TGF-β receptor mutations in LSD were found to be inactivating mutations (Horbelt et al. 2010; Loeys et al. 2006), which paradoxically leads to overactive TGF-β/Smad2 signaling.

Both MFS and LDS demonstrate an upregulation of TGF-β/Smad2 signaling. Consistent with the notion of overactive TGF-β signaling, neutralizing antibodies for TGF-β in animal models for MFS prevented the formation of fragmented elastin fibers and aortic aneurysms (Neptune et al. 2003). Importantly, losartan, an angiotensin inhibitor that inhibits TGF-β signaling through not well-understood mechanisms, also demonstrated a protective effect against aortic aneurysms (Habashi et al. 2006). Losartan has recently moved into phase III clinical trial in patients with MFS. Recently, the non-Smad signaling pathways, i.e., extracellular regulated kinase (ERK) and Jun N-terminal kinase (JNK) MAP kinases, were found to be elevated in MFS and to contribute to aortic aneurysms development (Holm et al. 2011). Selective inhibition of ERK or JNK in a MFS mouse model was found to inhibit aortic growth. Thus, inhibition of ERK and JNK may be beneficial for treatment of MFS.

While aortic aneurysms are one of the complications in MFS and LDS, familial thoracic aortic aneurysms and dissections can also result from mutations in these genes, resulting in loss of signaling through the TGF-β type I and II receptor, in the absence of all features of MFS and LDS (Milewicz et al. 1996; Pannu et al. 2005; Tran-Fadulu et al. 2009). Furthermore, mutations in *SMAD3* have been linked to the aneurysms osteoarthritis syndrome, a form of TAAD with tortuosity of the arterial tree and early onset of osteoarthritis (van de Laar et al. 2012; Regalado et al. 2011). In addition, the Shprintzen–Goldberg syndrome (SGS) that has considerable phenotypic overlap with MFS and LDS, including aortic aneurysm, was found to be linked with mutations in the proto-oncogene SKI, a known repressor of TGF-β activity (Doyle et al. 2012). Whether alterations in these TGF-β signaling components are inhibiting or enhancing TGF-β signaling is still a matter of debate.

13.3.3 TGF-β Signaling and Valvulopathies

TGF-β is crucial for valve development and homeostasis. The development of the valve starts with the formation of the cardiac cushion by EndMT of the endocardial cells. This occurs in the atrioventricular canal, which separates the atria and ventricles, and the outflow tract, which connects the ventricles with the aortic sac of the developing heart (Fig. 13.4) (Kruithof et al. 2012). Endocardial cushions are the primordia of the septa and valves and become mature structures by mesenchymal cell proliferation, remodeling, and valve elongation, resulting in thin leaflets with highly organized extracellular matrix. Valve maturation continues after birth before reaching the adult configuration with the mechanical properties to withstand the continuous changing of the hemodynamic environment during the heart cycle (Kruithof et al. 2012).

TGF-β is one of the key regulators of EndMT and an increased expression of TGF-β1 can be seen in the endocardial cells overlying the cushions, while TGF-β2 is expressed in the myocardium and endocardium flanking the cushions during initiation of cushion development (Akhurst et al. 1990; Molin et al. 2003). The

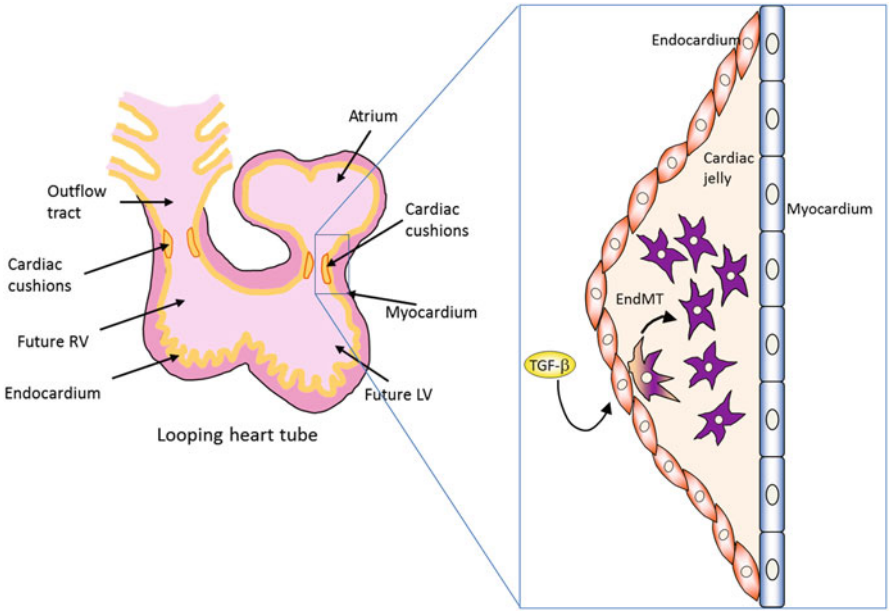

Fig. 13.4 Cardiac valve development. During embryogenesis the cardiac valves develop by the TGF-β-dependent mechanism of EndMT of the endocardial cells

requirement for TGF-β in cushion formation was initially shown using chicken explant cultures. When cultured in the presence of antisense oligo's or TGF-β neutralizing antibodies, EndMT, necessary for development of the cushions, was blocked (Boyer et al. 1999; Potts et al. 1991). Interestingly TGF-β1-deficient embryos, born to TGF-β1-null mothers, demonstrate severe cardiac abnormalities, including disorganized valves (Dickson et al. 1995). Furthermore, the valves in TGF-β2-deficient mice at E18.5 displayed defective remodeling and differentiation resulting in thickened valves (Azhar et al. 2011; Bartram et al. 2001; Sanford et al. 1997). The expression of TGF-β3 increases as valve development proceeds into advanced stages of heart development suggesting that TGF-β3 may play an important role in valve structure and function postnatal (Camenisch et al. 2002). Endocardium-specific deletion of ALK5 resulted in severe hypoplastic AV cushions, consistent with a crucial role for TGF-β signaling in EndMT (Sridurongrit et al. 2008). Endoglin is expressed in endocardial cells just before EndMT and endoglin deficiency results in embryonic lethality between E10.5 and E11.5 due to vascular abnormalities and disturbed cardiac development including hypocellular cushions (Arthur et al. 2000; Bourdeau et al. 1999; Li 1999). The TGF-β type III receptor betaglycan is also expressed in the endocardium during cushion formation (Stenvers et al. 2003), and betaglycan mutant mice die at the end of gestation harboring a thin ventricular wall and poorly developed septa (Compton et al. 2007).

Cardiac valve formation is crucial for proper heart function and septal/valve malformations comprise the largest part of congenital heart defects. Perturbation of TGF-β signaling pathway has been associated with both syndromic and non-syndromic congenital heart disease. Mutations in fibrillin-1, found in the genetic disorder marfan (see above), may cause thickening and elongation of the mitral valve leaflet, which can result in prolapse of the valve function (Judge et al. 2011). Mutations in Filamin-A, able to regulate TGF-β signaling, was found in patients with thickened valves (Kyndt et al. 2007). Furthermore, Williams syndrome, which has an elastin haploinsufficiency, shows elongated aortic valves, increased proliferation, and decreased TGF-β signaling (Hinton et al. 2010). Interestingly, a recent study, using a small molecule to inhibit ALK5 kinase activity, showed that indeed this receptor is important for the effect TGF-β has on valve formation. Using two independent chemical inhibitors lesions in the heart valves consisting of hemorrhages, inflammation, degeneration and interstitial cell activation, and proliferation were observed (Anderton et al. 2011). Most likely normal tissue repair is blocked as a consequence of ALK5 inhibition, and the valves may be particularly affected as a result of ongoing mechanical stress at opening and closure during each heart cycle.

13.3.4 TGF-β Signaling in Atherosclerosis

Atherosclerosis is a chronic inflammatory response in the arterial wall and characterized by vascular plaque formation (Libby et al. 2011). Stable plaques contain more collagen and smooth muscle cells, whereas unstable plaques have more macrophages and contain a large lipid core covered with a thin fibrous cap. Unstable plaques are more likely to rupture and cause clinical symptoms, e.g., cerebrovascular accidents or myocardial infarction. In atherosclerotic lesions, the TGF-β signaling components are detectable in endothelial cells, SMCs, myofibroblast, dendritic cells, T cells, and monocyte/macrophages and are rapidly upregulated during vascular injury (Bobik et al. 1999; Bot et al. 2009; Frostegård et al. 1999; Kalinina et al. 2004). There is some controversial information regarding the role of TGF-β in atherogenesis (Grainger 2004; Toma and McCaffrey 2012). Some reports show that increased TGF-β1 expression in human atherosclerotic plaques correlated with advanced atherosclerosis (Herder et al. 2012; Panutsopulos et al. 2005; Wang 1997), and other studies showed an inverse relationship between TGF-β1 levels in serum and the development of atherosclerosis, suggesting an anti-atherogenic effect (Grainger et al. 1995; Hering et al. 2002; Mallat et al. 2001). Furthermore, inhibition of TGF-β activity by various approaches resulted in pro-atherogenic changes in the vessel wall in animal models of atherosclerosis. Also an important role of TGF-β as an immune modulating cytokine in atherosclerosis was reported. Inhibiting TGF-β signaling in Apoe$^{-/-}$ mice by using a recombinant soluble TGF-RII (Lutgens 2002) or a blocking TGF-β1 antibody (Mallat et al. 2001) resulted in accelerated atherosclerosis. Lesions exhibited an unstable phenotype that contained low amounts of fibrosis, an increased amount of inflammatory cells, and even intraplaque hemorrhages.

Cardiac overexpression of TGF-β1, resulting in increased plasma levels of TGF-β, limited plaque growth and induced plaque stabilization (Frutkin et al. 2009). Mice with abrogated TGF-β signaling in T cells ($Apoe^{-/-}$CD4-dnTGF-βRII) (Gojova et al. 2003; Robertson et al. 2003) or DC cells ($Apoe^{-/-}$CD11cDNR) (Lievens et al. 2012) both showed accelerated lesion progression, with plaques containing abundant inflammatory cells paralleled by a decrease in plaque fibrosis.

It might be proposed that TGF-β signaling participates in the development of atherosclerosis, but may more interestingly promote a stable lesion phenotype, suggesting its role in the protection of acute ischemic situations like myocardial infarction. Interestingly, human aortic plaque vascular smooth muscles cells were shown to have enhanced levels of endoglin, supporting a role for endoglin in vascular wall integrity (Bot et al. 2009; Conley et al. 2000). In addition, soluble endoglin levels were found elevated in patients with coronary artery disease and atherosclerosis (Li et al. 2000b), and changes in soluble endoglin plasma levels post-myocardial infarction (MI) have predictive value for acute mortality in this patient group (Cruz-Gonzalez et al. 2008).

13.3.5 TGF-β Signaling in Cardiac Fibrosis

The development of heart failure starts with an acute MI or chronic injury, resulting in reduced ventricular performance and increased wall stress. Pathological deposition of extracellular matrix and myocardial hypertrophy are compensatory changes to reduce wall stress as the ventricle dilates. This will eventually lead to heart failure (Cohn et al. 2000). The process of cardiac fibrosis can be divided into two types: reactive fibrosis in response to inflammatory processes and reparative or replacement fibrosis in cell response to cell death and loss of myocardial tissue (Beltrami et al. 1994; Park et al. 2009; Silver et al. 1990). Cardiac fibroblasts are the interconnected cells that lie within the myocytes and extracellular space, and are the primary source of collagen in the heart (Souders et al. 2009). Activation of these fibroblasts or change of the fibroblast phenotype to myofibroblasts is what drives ECM accumulation and the development of pathological fibrosis. The local increase of TGF-β by various stimuli including inflammation, ischemia, mechanical stress, and vasoactive hormones like angiopoietin II is a key driver of this profibrotic process (Creemers and Pinto 2011; Dobaczewski et al. 2011; Goumans et al. 2008). Expression levels of TGF-β are increased in the left ventricle of both hypertrophic and idiopathic-dilated cardiomyopathy (Li et al. 1997; Martin et al. 2005; Pauschinger et al. 1999; Villarreal and Dillmann 1992), and in the border-zone of the infarcted region following myocardial infarction (Chuva de Sousa Lopes et al. 2004; Frantz et al. 2008) and due to diabetes (Connelly et al. 2009; Westermann et al. 2007).

That indeed TGF-β promoted myocardial hypertrophy and fibrosis was shown by overexpressing TGF-β1 in mice (Rosenkranz et al. 2002). These animals developed significant cardiac hypertrophy accompanied by interstitial fibrosis. In addition,

TGF-β1 heterozygous animals were protected from age-associated cardiac fibrosis and diastolic dysfunction (Brooks and Conrad 2000). Furthermore, treating animals with an anti-TGF-β neutralizing antibody prevented collagen accumulation following pressure overload and attenuated diastolic dysfunction without affecting cardiac hypertrophy (Ellmers et al. 2008; Kuwahara et al. 2002; Okada et al. 2005). Interestingly, mice overexpressing a dominant negative TGF-βRII showed markedly reduced collagen deposition following pressure overload, resulting in increased left ventricular dilatation and diastolic dysfunction (Lucas et al. 2010; Okada et al. 2005). Endoglin is also expressed in cardiac fibroblast and found to be increased during Ang-2-induced cardiac fibrosis. Recently, Kapur and coworkers showed that when treating animals with soluble endoglin 1 day before aortic bending, cardiac fibrosis and the development of heart failure were reduced (Kapur et al. 2012).

The functional effects of TGF-β signaling are likely to be biphasic. TGF-β signaling is necessary to preserve cardiac structure to protect the heart from the increased pressure and uncontrolled matrix degradation, while excessive TGF-β signaling results in enhanced collagen deposition, increased myocardial stiffness, and diastolic dysfunction. That TGF-β may function as a master switch is nicely demonstrated by inhibiting TGF-β signaling post MI. Early TGF-β inhibition within 24 h after occlusion of the coronary artery enhanced the inflammatory response and increased mortality (Frantz et al. 2008; Ikeuchi et al. 2004), while late TGF-β inhibition after the inflammatory phase of infarct healing reduced the number of myofibroblasts and improved cardiac output (Okada et al. 2005).

Both the intracellular Smad signaling pathways as well as non-Smad pathways are thought to play a role in the pathological remodeling of the heart. Smad3 was shown to be involved in myofibroblast transformation and mediated the TGF-β-induced ECM production and tissue inhibitor of matrix metalloprotease (TIMP) upregulation (Dobaczewski et al. 2010). In addition, TGF-β-activated kinase (TAK1) is activated in the pressure overloaded myocardium, and activated TAK expressed in the mouse myocardium induces cardiac hypertrophy and severe systolic dysfunction (Zhang et al. 2000). Finally, an indirect effect of TGF-β on cardiac fibrosis, by inducing, e.g., connective tissue growth factor (CTGF) and endothelin (Leask 2010), cannot be excluded.

13.4 Concluding Remarks

TGF-β plays a pivotal role in controlling cardiovascular homeostasis. Mutations in human TGF-β signaling components such as TGF-β receptors and Smads have been directly linked to cardiovascular disorders. Moreover, genetic studies in mouse models revealed that too little or overactive TGF-β signaling in ECs and SMCs leads to vascular dysfunction. Several small molecular weight compounds and antibodies that modulate TGF-β or BMP signaling are being evaluated in (pre)clinical trials for cardiovascular disorders. Examples are: losartan to inhibit overactive TGF-β signaling in Marfan syndrome (Möberg et al. 2012), Bevacizumab

(a VEGF-A neutralizing antibody) and thalidomide to normalize and stabilize the weak vessel phenotype of HHT patients (Dupuis-Girod et al. 2012; Lebrin et al. 2010), and TGF-β receptor kinase inhibitors to correct the BMP/TGF-β imbalance seen in pulmonary hypertension (Long et al. 2009). However, with TGF-β being such a multifunctional model with effects on many different cell types that are highly context dependent, TGF-β signaling components remain very challenging therapeutic targets. Current treatments that target TGF-β or its receptors are not selective for only the pathological signaling pathways triggered by TGF-β. Careful selection and monitoring of patients are needed to guard for side effects.

There remains to be urgent need to more precisely dissect the mechanisms of TGF-β in vascular cells and identify cell type-specific regulators of TGF-β signaling, thereby enabling strategies to selectively target "bad" responses, while leaving "good" effects of TGF-β intact. The use of conditional knockouts and knockins for TGF-β signaling components in different cells, different tissues, and different times will be very instrumental to achieve this. Moreover, induced pluripotent stem cells (iPSCs) technology may be used to generate cardiovascular patient-derived endothelial and smooth muscle cells (Reed et al. 2012). These cells can be used not only to study pathology, but can also be valuable as tools to identify small molecule drugs that rescue the disease cell phenotype. Advances in all the above research fields will provide new opportunities for treatment of increasing number of vascular pathologies that are associated with dysregulation in TGF-β signaling.

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Chapter 14
TGF-β Signaling in Physiological and Pathological Angiogenesis

Sara I. Cunha and Kristian Pietras

Abstract Members of the transforming growth factor-β (TGF-β) family exert their effect virtually on all cell types in the body, producing diverse and complex cellular responses. TGF-β signaling is deregulated and hyperactive in many malignant conditions, making it an appealing target in the combat of cancer disease. The predominantly endothelial TGF-β receptors, ALK1 and endoglin, which are activated during neoangiogenesis both during development and pathological conditions, pose attractive modulating opportunities to impair tumor vessel formation and cancer progression. However, the precise function of TGF-β family signaling in ECs is difficult to predict, as it appears highly context dependent due to the many ligands and receptors influencing the final outcome. Furthermore, TGF-β is involved in autocrine and intricate dynamic paracrine signaling events in the context of the tumor microenvironment. Pharmacological inhibitors for ALK1, endoglin, and TGF-β or its receptors have been developed and will facilitate more comprehensive studies on the exact function of the TGF-β family in the endothelium, and more specifically in tumor angiogenesis. Here, we will summarize the current knowledge on TGF-β signaling in the regulation of the formation and function of the vascular network.

Keywords Angiogenesis • BMP • Cancer • Targeted therapy • TGF-β

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Abbreviations

ALK	Activin receptor-like kinase
AVM	Arteriovenous malformations
BMP	Bone morphogenetic proteins
EC	Endothelial cell
EMT	Epithelial-to-mesenchymal transition
EndMT	Endothelial-to-mesenchymal transition
FGF	Fibroblast growth factor
GDF	Growth and differentiation factor
HHT	Hereditary hemorrhagic telangiectasia
MMP	Matrix metalloproteinase
TGF-β	Transforming growth factor-β
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell

14.1 Introduction

14.1.1 The TGF-β-Rich Tumor Microenvironment

The tumor microenvironment, or stroma, influences the growth of the tumor and its ability to progress and metastasize. The stroma thus constitutes an important aspect to consider when developing therapeutic approaches, as it can alter interstitial fluid pressure, limit the access of therapeutics to the tumor, change drug metabolism, or even contribute to the development of drug resistance (Egeblad et al. 2010).

Despite the importance of tumor–host stroma interactions, there is limited understanding of the stromal milieu composition, and the complexity and dynamics in the relationship between the tumor malignant cells and the surrounding host cells. It is, however, acknowledged that tumor cells and their stroma coevolve during tumorigenesis and progression (Pietras and Östman 2010). Nonetheless, the precise nature of the cells that comprise the normal stroma, how these cells or newly recruited cells are altered during tumor progression, and how they reciprocally influence tumor initiation and progression, is poorly understood.

The large family of TGF-β extracellular pleiotropic cytokines exerts influence essentially on all cellular strata in the body, namely in epithelial cells, fibroblasts, immune, endothelial, lymphatic and perivascular cells (Fig. 14.1) (Elliott and Blobe 2005). TGF-β is the prototypical element of an extensive ligand group that also includes bone morphogenetic proteins (BMPs), activins, inhibins, nodal, and growth and differentiation factors (GDF)s that elicit signaling activity through a collection of five type II receptors and seven type I receptors (Padua and Massague 2009). Different ligand-receptor II–receptor I combinations can be assembled, hence delineating one of the TGF-β family hallmarks of complexity.

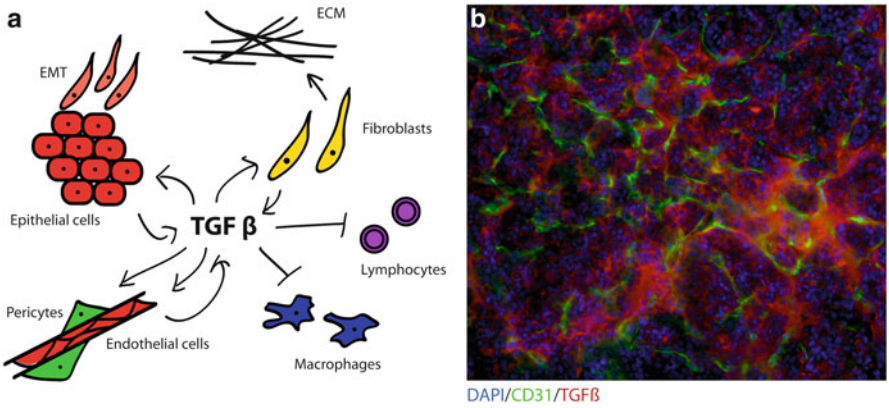

Fig. 14.1 (**a**) TGF-β secreted from various sources is acting on a wide range of cell types in the tumor microenvironment. TGF-β promotes tumor epithelial cell invasiveness and metastasis in an autocrine fashion. TGF-β also affects tumor matrix remodeling by cancer-associated fibroblasts and regulates angiogenesis by acting on ECs and pericytes. Tumor infiltration by leukocytes, macrophages, and bone marrow-derived endothelial, mesenchymal, and myeloid precursor cells is also mediated by TGF-β. TGF-β suppresses proliferation and differentiation of lymphocytes including T cells, natural killer cells, and macrophages, preventing immune surveillance control over the developing tumor. (**b**) Immunostaining of an experimental pancreatic neuroendocrine tumor from RIP1-Tag2 mice for TGF-β (*red*) and the endothelial cell marker CD31 (*green*) illustrates the abundance of TGF-β throughout the tumor microenvironment

In normal, unstressed tissue, sustained basal release of TGF-β by local sources regulates homeostasis. In pathological conditions, TGF-β is abundantly released in the tumor microenvironment (Fig. 14.1), initially as a signal to avoid premalignant progression, but eventually as a cue that cancer cells utilize to their own advantage in later stages of malignancy (Massague 2008; Wakefield and Roberts 2002). Most human tumors overproduce TGF-β whose autocrine and paracrine actions promote tumor epithelial cell invasiveness and metastasis, functioning as a differentiation switch required for transient but reversible invasiveness of carcinoma cells through epithelial-to-mesenchymal mechanisms (Pardali and Moustakas 2007). In addition to eliciting mitogenic signals toward the carcinoma cells, TGF-β also affects cancer-associated fibroblast induction of tumor matrix remodeling and regulates angiogenesis by acting on endothelial cells (ECs) and pericytes. Finally, TGF-β suppresses proliferation and differentiation of lymphocytes including T cells, natural killer cells, and macrophages, thus preventing immune surveillance control over the developing tumor.

In a nutshell, TGF-β signaling is intimately implicated in tumor development and contributes to most hallmarks of cancer described by Hanahan and Weinberg (2000, 2011). It is, thus, of vital importance to carefully analyze the role of the TGF-β family members in the tumor microenvironment and how signaling circuits arising from tumor and stromal interactions can be efficiently modulated in cancer therapy.

14.1.2 Involvement of TGF-β in Vascular Syndromes

In vascular biology, TGF-β has traditionally been seen as a differentiation regulator for vascular smooth muscle cells (VSMCs), ultimately contributing to vessel stabilization and maturation, by inducing ECM deposition and inhibiting EC migration and proliferation.

The critical relevance of TGF-β signaling in vascular development was, however, recognized by identification of mutations in TGF-β receptor genes in familial vascular syndromes, in the type I endothelial specific receptor, activin receptor-like kinase (ALK)1, and the type III or co-receptor, endoglin (ten Dijke and Arthur 2007). Germline loss-of-function mutations in ALK1 or endoglin are causal in the development of the human syndrome of hereditary hemorrhagic telangiectasia (HHT). HHT is characterized by cutaneous telangiectases and gastrointestinal hemorrhage (Berg et al. 1997; McAllister et al. 1994). In addition, major arteriovenous malformations occur in lung, liver, or brain, which may ultimately cause severe morbidity and mortality. While *Alk1* and *endoglin* null mice die at midgestation as a result of severe arteriovenous malformations (AVMs) (Arthur et al. 2000; Oh et al. 2000), mice lacking one copy of the gene for either *Alk1* (Alk1+/–; HHT2) or *endoglin* (Eng+/–; HHT1) recapitulate with age the HHT phenotype (Srinivasan et al. 2003; Torsney et al. 2003).

There is additional strong evidence gathered from in vivo studies on genetically manipulated mouse models, for a prominent role of the TGF-β pathway in vasculo- and angiogenesis mechanisms (ten Dijke and Arthur 2007).

Nearly half of TGF-β1$^{-/-}$ and 25 % of TGF-β1+/– mouse embryos die in utero due to defective hematopoiesis and vasculogenesis (Dickson et al. 1995). TGF-β1-deficient mice that do survive until birth die at about 3 weeks of age of a multifocal inflammatory disorder, primarily affecting heart and lungs, although mild liver inflammation has been also observed (Shull et al. 1992). Supporting these reports, the classical TGF-βRII and TGF-βRI/ALK5 receptors also exhibit defective vasculogenesis and lead to mid gestation lethality (Larsson et al. 2001; Oshima et al. 1996).

In fact, deletion of TGF-β signaling mediators results (Table 14.1), for most cases, in serious implications on the developing embryonic vasculature leading ultimately to lethality in mice (Bertolino et al. 2005; ten Dijke and Arthur 2007).

A lot of attention has been focused on the endothelial cell-specific TGF-β receptors ALK1 and endoglin not only because they already have documented involvement in the human syndrome HHT, but more importantly they hold therapeutic promise in pathological conditions, such as cancer, where their upregulated expression in the vascular component plays a critical role in cancer development.

Table 14.1 TGF-β signaling mediators implicated in vascular abnormalities in mice and human pathological conditions

Gene	Mouse phenotype	Human pathological condition	References
TGF-β1	Null: embryonic lethality with angiogenesis defects; some are born and die after a few weeks due to inflammatory disorders		Shull et al. (1992), Dickson et al. (1995)
TGF-β2	Null: aortic arch defects, cardiac septal defects, abnormal cardiac cushions, persistent truncus arteriosus, perinatal lethality		Shull et al. (1992), Sanford et al. (1997)
BMP 2	Null: embryonic lethal with defects in amnion/ chorion and cardiac development.		Zhang and Bradley (1996)
	Het: viable; susceptible to hypoxic pulmonary hypertension		Anderson et al. 2010)
BMP 4	Het: viable; less severe hypoxic pulmonary hypertension; impaired vascular remodeling		Anderson et al. (2010)
TGF-βRI/ALK5	Null: embryonic lethal, angiogenesis defects	Aortic aneurysm; LDS	Larsson et al. (2001)
TGF-βRII	Null: embryonic lethal with defects in vasculogenesis and hematopoiesis	MFS2, LDS	Oshima et al. (1996)
BMPRII	Null: pre-angiogenesis lethality	PAH	Beppu et al. (2004), Song et al. (2005)
	Het: Susceptible to pulmonary hypertension, defective vascular remodeling, abnormal vascular tone		Rudarakanchana et al. (2002)
TβRIII	Embryonic lethal at E13.5 with proliferative defects in the heart and apoptosis in the liver; defects in coronary vasculogenesis		Stenvers et al. (2003), Compton et al. (2007)
ALK1	Null: Embryonic lethal at E11.5 with angiogenesis defects; dilated vessels, AVMs, impaired VSMC recruitment	HHT	Oh et al. (2000) Urness et al. (2000)
	Het: model for HHT		Park et al. (2006)

(continued)

Table 14.1 (continued)

Gene	Mouse phenotype	Human pathological condition	References
	EC-specific deletion: vascular dysplasia, susceptibility to arteriovenous malformations		
ALK2	Null: lethality, defects in cardiovascular embryogenesis, nervous system, skeleton, growth and size; impaired bicuspid aortic valve	FOP	Shore et al. (2006) Thomas et al. (2012)
Endoglin	Null: Embryonic lethal with angiogenesis and cardiac defects	HHT	Arthur et al. (2000) Bourdeau et al. (2000)
	Het: model for HHT, spontaneous pulmonary hypertension, abnormal vascular tone		Mahmoud et al. (2010)
	Conditional deletion in EC: arteriovenous malformations		
ALK3/BMPRIa	Conditional deletion in the mesoderm: embryonic lethal at E10.5 with hemorrhage, cardiac defects, and impaired smooth muscle cell recruitment		Park et al. (2006)
	Conditional deletion in embryonic SMCs: embryonic lethality at E11.5 with cardiac defects, hemorrhage, impaired vascular remodeling		El-Bizri et al. (2008a), El-Bizri et al. (2008b)
	Conditional deletion in adult SMCs: viable with impaired vascular remodeling		
Smad 1	Null: embryonic lethal at E9.5 with defects in fusion of the chorioallantoic membrane and yolk sac angiogenesis		Lechleider et al. (2001)
Smad 2	Null: pre-angiogenesis lethality		Goumans and Mummery (2000)
Smad 4	Null: pre-angiogenesis lethality	Juvenile polyposis; HHT	Gallione et al. (2004)

(continued)

Table 14.1 (continued)

Gene	Mouse phenotype	Human pathological condition	References
	Conditional deletion in EC: embryonic lethal at E10.5 with cardiac and angiogenesis defects		Lan et al. (2007)
Smad 5	Null: embryonic lethal at E9.5 with cardiac/ angiogenesis defects		Chang et al. (1999), Yang et al. (1999)
Smad 6	Null: some viability; cardiac defects, vascular calcification and systemic hypertension		Galvin et al. (2000)
Smad 8	Null: viable with spontaneous pulmonary vascular remodeling		Huang et al. (2009)
EMILIN 1	Null: reduced arterial diameter, increased vascular resistance, emphysema, increased TGF-β signaling in the vascular wall	Hypertension	Zacchigna et al. (2006)
Fibrilin 1	Null: die perinatality with ruptured aortic aneurysm, impaired pulmonary function	MFS1	Carta et al. (2006), Judge et al. (2004)
Fibronectin 1	Null: deformed heart and embryonic vessels, defective extraembryonic vasculature	Ehlers–Danlos syndrome	George et al. (1993)
LTBP1	Null: persistent truncus arteriosus and cardiac valve development		Todorovic et al. (2007)
Thrombospondin 1	Null: pneumonia, alveolar hemorrhage, VSMC hyperplasia		Lawler et al. (1998), Crawford et al. (1998)

HHT hereditary hemorrhagic telangiectasia, *LDS* Loeys–Dietz syndrome, *MFS2* Marfan syndrome type 2, *PAH* pulmonary arterial hypertension, *vSMC* vascular smooth muscle cell, *FOP* fibrodysplasia ossificans

14.1.3 TGF-β Signaling in ECs: ALK1/ALK5 Interconnecting Crosstalk

Following synthesis, secretion, and activation, the mature TGF-β dimeric ligand is released from the ECM to trigger specific serine/threonine type I and type II kinase receptor heterotetrameric complexes (Wrana et al. 1994). In ECs, TGF-β signaling has been described to signal via the globally expressed type I receptor, ALK5 or alternatively through the predominantly endothelial type I receptor, ALK1.

The prevailing recruited type I receptor dictates the activation of a particular Smad transducing cascade. ALK1 activation generates phosphorylation of Smad1, 5, and 8, whereas ALK5 leads to Smad 2 and 3 signaling activation (Goumans et al. 2002). The selected activated Smad subset independently forms a heteromeric complex with a related molecule, Smad4, which translocates the complexes into the nucleus to launch transcription of specific target genes (Massague 2000; ten Dijke et al. 2000), involved in distinct angiogenic responses.

TGF-β ligands also interact with co-receptors or type III receptors, represented by betaglycan (TGF-βRIII) and the typically endothelial endoglin. However, because these type III receptors lack the kinase domain, they essentially hold an accessory role in ligand binding and signaling activation, adding yet another level of regulation to the TGF-β complex signaling web.

Far from consensual, the TGF-β contribution to vascular biology knowledge has been constantly under debate due to numerous paradoxical reports (Goumans et al. 2002; Lamouille et al. 2002; Valdimarsdottir et al. 2002).

A crucial role in angiogenesis for ALK1 was first described in a study reporting ALK1 as pivotal for SMC recruitment, implying a vital role for TGF-β/ALK1 signaling axis in the maturation phase of angiogenesis (Oh et al. 2000). On the other hand, signaling derived from ALK5 was more pronounced during the activation phase of angiogenesis, when ECs degrade perivascular basement membrane, invade and migrate into the newly available space, through active proliferation and lumen formation. The balance theory was then hypothesized for the first time, speculating that different levels of TGF-β ligand availability would determine the sequential angiogenic fate and control the properties of the endothelium during angiogenesis. Also, in parallel studies another laboratory presented evidence for development of shunts between arteries and veins and severe arteriovenous malformations due to fusion of major arteries and veins in mice lacking ALK1 (Urness et al. 2000).

The balance working model was quickly challenged when another laboratory proposed that TGF-β engages in the activation of ALK1 signaling via Smad1/5, which concomitantly inhibits ALK5 signaling through Smad2/3 (Goumans et al. 2002). ALK5, while critical for ALK1 signaling, demonstrated by studies on ALK5-deficient mouse embryonic ECs, commits to an anti-angiogenic cascade of events, while ALK1 mediates pro-angiogenic activation (Goumans et al. 2003). These studies indicated that TGF-β stimulatory effects on either ALK5 or ALK1 are mutually exclusive inducing differential transcriptional activation of PAI-1 and Id1, respectively, which ultimately elicit a different set of physiological responses.

Motivated by the fact that both ALK1 and ALK5 null mice render an embryonic lethal phenotype due to extensive vascular abnormalities (Larsson et al. 2001; Oh et al. 2000; Urness et al. 2000), ALK1 dependency on ALK5, by means of signaling or by mere anchoring, has been questioned and addressed with reservation by multiple laboratories.

Transcriptional profiling of human umbilical vein ECs expressing constitutively active adenoviral constructs of ALK1 or ALK5 demonstrated substantial differences

in the transcriptional output from either signaling pathway (Ota et al. 2002), validating previously described downstream gene regulation. Interestingly, the non-overlapping expression patterns of ALK1 and ALK5 in vivo (Seki et al. 2006) by thorough analysis of a knockin mouse line carrying a lacZ reporter in the *Alk5* gene locus ($Alk5^{lacZ}$), also lends support to divergent roles in vascular development for each of the two type I receptors expressed by ECs.

ALK5 suppression, by genetic silencing or small molecule inhibition, was shown not to interfere with BMP9/ALK1-induced phosphorylation of Smad1/5/8 in bovine aortic ECs (BAECs) (Shao et al. 2009). Instead, silencing of Alk1 or any of its downstream molecular effectors, by means of siRNA transfection, rather induce a potent ALK5 signaling upregulation. In agreement with the ALK5-independent action of ALK1 is the notion that ALK5 is present in ECs in vivo either at low levels, or only expressed by the neighboring VSMCs, suggesting that ALK5 may only participate in ALK1-dependent angiogenesis in a paracrine fashion (Park et al. 2008; Shao et al. 2009). Congruent with these results, EC-specific ablation of Alk5 does not inflict vascular abnormalities in mice or zebrafish (Park et al. 2008). However, embryos from knockin mice carrying a mutation on L45 loop in Alk5 rescued to some extent the earliest vascular defects observed in ALK5 mouse knockouts (Itoh et al. 2009), probably because this mutation, yet interfering with ALK5 kinase ability to phosphorylate Smad2, inherently preserves ALK5 competence to mediate non-Smad signaling and lateral signaling to ALK1. In agreement with these findings, ALK1 signal inhibition proved to interfere, in pathological conditions, not only with its own target genes but ALK5 signal transduction also exhibited a suppressive modulation in a model of pancreatic neuroendocrine cancer (Cunha et al. 2010).

More recently, it has been demonstrated that selective deletion of ALK5 in ECs using an $Alk5^{GFPCre}$ mouse line resulted in embryonic lethality due to brain vessel pathological morphology and intracerebral hemorrhage (Nguyen et al. 2011). Independent observations of EC-specific deletion of Smad2/3 using Tie2-Cre transgenic mice revealed critical hemorrhaging and embryonic lethality around E12.5. In this study, vascular maturation was incomplete owing to inadequate assembly of mural cells in the vasculature, most likely because of impaired expression of PDGF-B by the Smad2/3 ablated endothelium (Itoh et al. 2012). These observations substantiate the vital relevance of ALK5 in the endothelium either as a signaling anchor or by actively participating in the vasculogenic process.

Collectively, these reports demonstrate that ALK5 signaling is indeed relevant for endothelial homeostasis. Further studies aiming at dichotomizing ALK1 versus ALK5 signaling in endothelial and in perivascular cells during development and in the tumor microenvironment are thus required to clarify and reconciliate previous paradoxical observations and infer about the benefit or risk of clinically targeting such pathways without proper amendments. It is plausible that the relative stoichiometry of ALK1 and ALK5 signaling may be crucial for proper regulation of gene expression (Cunha and Pietras 2011).

14.1.4 Bone Morphogenetic Proteins

The BMPs, a subcategory of the TGF-β superfamily, were first identified in extracts from bone matrix and characterized by their ability to induce ectopic bone formation when implanted subcutaneously in rats (Wozney et al. 1988). It soon became clear that BMPs play a key role in vertebrate organogenesis, as well as in embryonic vascularization (Kishigami and Mishina 2005; Zhao 2003).

The BMP family, including the GDFs, comprises a group of 20 ligands that activate a classical BMP pathway in vertebrates (Lowery and de Caestecker 2010; Wagner et al. 2010). In the canonical BMP signaling pathway, three type II receptors (BMPRII, ActRIIa, and ActRII2b) and four type I receptors (ALK1, ALK2, ALK3, and ALK6) can be activated (Miyazono et al. 2010; Moustakas and Heldin 2009). In addition to primarily triggering Smad1, 5, and 8, BMP cues may also activate Smad2 (Upton et al. 2009) and Smad-independent signaling (Sieber et al. 2009).

BMP9 has been implicated in hematopoiesis, hepato-, osteo-, chondro-, and adipogenesis (Canalis et al. 2003; Li et al. 2003; Lord et al. 2010; Ploemacher et al. 1999; Sieber et al. 2009). It has also been described as a regulator of glucose metabolism (Chen et al. 2003) and as a differentiation factor for cholinergic neurons in the central nervous system (Lopez-Coviella et al. 2000). More recently, BMP9 was pinpointed as the physiologically functional high affinity ligand for the predominantly endothelial receptor, ALK1. This fact highlighted BMP9 as a critical modulator of angiogenesis (David et al. 2007, 2008; Scharpfenecker et al. 2007).

BMP9 was originally cloned from a rodent cDNA library obtained from mouse liver, where it was shown to be highly expressed (Miller et al. 2000). Accordingly, the liver was characterized as the major source of human and mouse BMP9, expressed by hepatocytes and intrahepatic biliary epithelial cells, while brain and lung only express it at much lower levels (Bidart et al. 2012). In line with these observations, the Human Protein Atlas profile for BMP9 in normal tissues indicates that it is highly expressed in liver, pancreas, placenta, lung, epididimus, gastrointestinal tract, gall bladder, and thyroid, but also in hematopoietic cells (www.proteinatlas.org).

Interestingly, it is now clearly described that pulmonary and cerebral arterial malformations occur more often in HHT1, while hepatic arterial malformations are more frequent in HHT2 (Letteboer et al. 2006). In fact, in HHT2, the frequency of hepatic arterial malformations is between 38 % and 41 %, while in HHT1, it ranges between 2.5 % and 8 % (Gallione et al. 2006). The specific expression of the ALK1 ligand, BMP9, predominantly in the liver reflects a seemingly tissue-specific manifestation in HHT2.

The *modus operandus* of BMP ligand interaction with their receptors differs from that of TGF-β: while TGF-β exhibits higher affinity for type II receptors and do not stably interact with type I receptors alone, BMPs bind independently to both type I and type II receptors (Groppe et al. 2008; Lin et al. 2006). The BMP ligands can also display affinity to the co-receptors endoglin and betaglycan (Lowery and de Caestecker 2010). In fact, BMP9 can directly bind endoglin (Scharpfenecker et al. 2007).

BMP9 is synthesized as a precursor protein, which is then cleaved by furin, a serine-endoprotease, forming a short dimeric mature form to which the prodomain can remain non-covalently associated (Bidart et al. 2012). Until recently, neither BMP9 nor its closely related family member BMP10 was found to be negatively regulated by common BMP pathway antagonists (David et al. 2008; Seemann et al. 2009). However, recent studies show that ALK1 activation by BMP9 induces expression of matrix Gla protein and crossveinless 2 (CV2), both known as antagonists of BMP4-induced angiogenesis (Yao et al. 2006, 2011, 2012). CV2, a member of the Chordin family, preferentially binds and inhibits BMP9 thereby providing strong feedback inhibition on ALK1 (Yao et al. 2012), suggesting a critical mutual regulation by BMP9 and CV2 in vasculature regulation.

Analogous to TGF-β signaling mediated by ALK1, BMP9 has also been reported to have incongruent effects on ECs. For example, BMP9 exhibits anti-angiogenic effects counteracting fibroblast growth factor (FGF)-induced angiogenesis in ex vivo metatarsal models (David et al. 2007; Scharpfenecker et al. 2007) and acts as a circulating vascular quiescent factor (David et al. 2008). Nevertheless, multiple types of ECs activate their proliferative status in vitro in response to BMP9, which pro-angiogenic properties also activate matrigel plug vascularization and tumor angiogenesis in a pancreatic cancer xenograft model (Suzuki et al. 2010).

In order to unmistakably clarify the effects on the endothelium by BMP9 stimulation and its specific downstream mediators, an extensive analysis of BMP9 downstream activation in comparison to other ligands on ECs is mandatory in the field. According to the present knowledge, BMP-induced responses have as common denominator the Smad1/5/8 pathway and Id1, 2, and 3 as target genes, suggesting other differentially responsive genes may exist, more specifically induced by each BMP. In a recent study such efforts were initiated where EC-specific Smad1/5 target genes were characterized and upregulation of Notch signaling-related genes were identified upon BMP9 stimulation (Morikawa et al. 2011).

Despite the paucity of detailed studies, the Human Protein Atlas profile for BMP9 in cancer disease indicates that BMP9 expression is increased in colorectal cancer, head and neck squamous carcinoma, and pancreatic and liver cancers (www.proteinatalas.com). Interestingly, BMP9 is primarily expressed in the islets of Langerhans by the tumor cellular compartment in mouse neuroendocrine pancreatic tumors (Cunha et al. 2010).

Similar to BMP9, BMP10 has also been identified as a functional activator of ALK1 in ECs, inducing comparable cellular effects. In agreement with a 65 % amino acid sequence homology between both ligands, BMP10, much like BMP9, exhibits angiostatic properties on dermal HMVEC (David et al. 2007). Nevertheless, BMP10 binds to ALK1 with lower affinity than BMP9 (David et al. 2007) and is mainly expressed in the murine developing and postnatal heart. The impaired lethal cardiac growth and physiology in the BMP10 knockout mouse coupled to normal vascular development of embryo and yolk sac propose a critical role for BMP10 in cardiogenesis (Chen et al. 2004). Interestingly, it has also been demonstrated that BMP10 can additionally bind to ALK3 (Mazerbourg et al. 2005). Of note, ALK3 targeted deletion in neural crest cells generates embryonic heart failure (Stottmann et al. 2004).

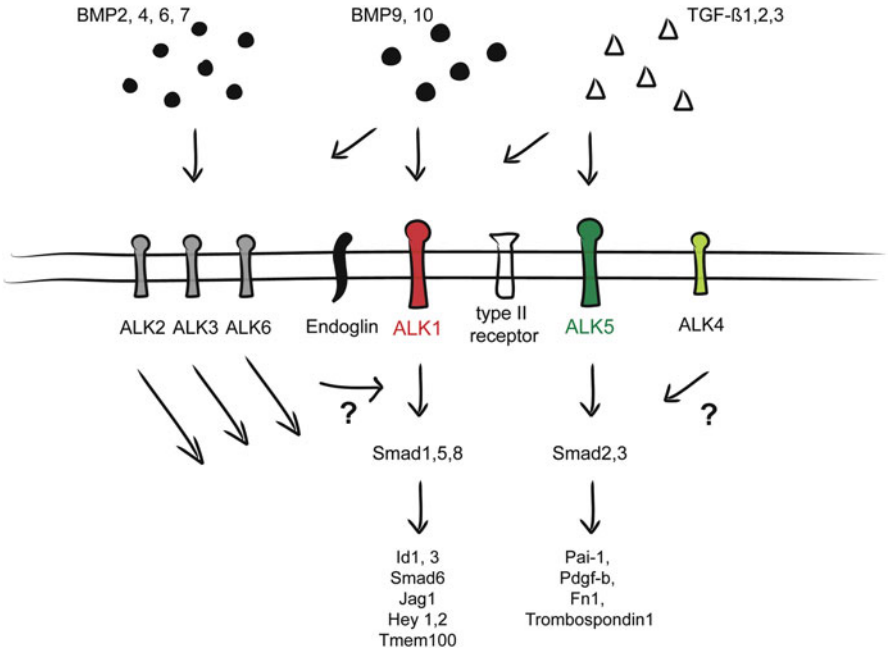

Fig. 14.2 Multiple TGF-β family ligands and receptors are acting on endothelial cells to shape the angiogenic response

All in all, these observations suggest the cardiac-specific nature of BMP10 signaling most likely through ALK3, rather than ALK1. However, the direct effect of BMP10 on the vasculature should not be overlooked, as it has potential to cooperate or even compensate for BMP9 signaling through ALK1. In fact, very recently Ricard et al. unveiled that *Bmp9*-KO mice do not exhibit defective vascularization in the retina (Ricard et al. 2012). However, injection of the extracellular domain of ALK1 or a neutralizing anti-BMP10 antibody impaired retinal vascularization in Bmp9-KO neonates, reducing retinal vascular expansion and exacerbating vascular density (Ricard et al. 2012). These data thus sustain a cooperative or compensatory role for BMP9 and BMP10 in postnatal vascular remodeling of the retina. Whether this cooperative role occurs in the context of cancer remains to be determined.

14.1.5 Interplay Between Type I Receptors

As mentioned earlier, ALK1 shares similar properties in terms of BMP-dependent activation of Smad1/5/8 signaling with the related BMP type I receptors ALK2, ALK3, and ALK6 (Fig. 14.2). Ligand specificity has not been carefully elucidated, and many ligands, including BMP2, BMP4, BMP6, BMP7, BMP9, and BMP10,

exhibit a multitude of effects on ECs, ranging from metabolism, endothelial-to-mesenchymal transition (EndMT), and tumor angiogenesis (Fig. 14.2) (Bostrom et al. 2011; Heinke et al. 2008; Langenfeld and Langenfeld 2004; Medici et al. 2010; Ramoshebi and Ripamonti 2000; Yao et al. 2008; Zeisberg et al. 2007). While described as the physiological ligand for ALK1, BMP9 has also documented binding ability toward ALK2 in non-EC, such as myoblasts and breast tumor cells (Scharpfenecker et al. 2007), with the BMP9/ALK2 signaling axis being also linked to promotion of proliferation of ovarian cancer cells (Herrera et al. 2009). Lending support to the need of substantial analysis of the signaling arising from these receptors in the endothelium, vascular ECs have been shown to transform into multipotent stem-like cells in an ALK2-dependent fashion, in lesions from individuals with *fibrodysplasia ossificans progressiva* (FOP) (Medici et al. 2010). This disabling disorder occurs as a result of gain-of-function mutations in ALK2 in humans or mirrored in mice by constitutive activation of ALK2 signaling on chondrocytes and osteoblasts. Lineage tracing of heterotopic ossification in mice using a Tie2-Cre construct disclosed the endothelial origin of these cell types (Medici et al. 2010). In agreement with this finding, ECs conditionally deficient for ALK2 do not succeed to undergo EndMT during endocardial cushion formation in embryogenesis (Wang et al. 2005). Of note, ALK2 has been demonstrated to upregulate ALK1 in ECs in response to high-density lipoproteins, after which ALK1 in turn promotes survival by inducing expression of vascular endothelial growth factor (VEGF)-A (Yao et al. 2008). Glucose level augmentation co-regulates ALK1 and ALK2 expression in human aortic ECs (Bostrom et al. 2011). Also, BMP/TGF-β receptors appear to be activated and function sequentially: ALK3, ALK2, ALK1, and ALK5, where each receptor can possibly entail a distinct function and correlate to a specific stage in vascular growth and development (Bostrom et al. 2011; Shao et al. 2009; Yao et al. 2008).

Thus, the interplay and/or compensatory crosstalk primarily between ALK1 and ALK2, but also with ALK3 and ALK6, which is of critical importance in a therapeutic context, promptly begs for more detailed tumor studies.

14.1.6 TGF-β and BMP Signaling Pathways: Competitive or Synergistic?

Closely connected to receptor interplay is the role specifically played by the ligands. Classically, BMPs and TGF-βs have long been described to exert parallel antagonistic effect on the other pathway in a variety of biological contexts (Gronroos et al. 2012).

Moreover, in physiological conditions, cells in the body are exposed to multiple ligands simultaneously, which may render alternative responses than what is customarily studied when analyzing the effects of each ligand in isolation. In an attempt to clarify the role of ALK1 signaling in EC, we recently described an unanticipated synergistic effect of TGF-β with BMP9 on tumor angiogenesis. We demonstrated in vitro and in vivo in various systems, that while either cytokine on its own exerted suppressive action on the endothelium, both ligands in combination boosted the EC

response toward other pro-angiogenic stimuli (Cunha et al. 2010). On a molecular level, simultaneous ECs induction with TGF-β and BMP9 induces a synergistic response on ALK5 target gene expression (e.g., PAI-1 and PDGF-B).

Another publication demonstrated that BMP2 synergistically enhances TGF-β3-induced initial phenotypic changes associated with EndMT, taking place during endocardial cushion formation (Yamagishi et al. 1999). Of note, BMPs and their receptors are expressed at many sites in which epithelial or endothelial-to-mesenchymal transition occurs during developmental organogenesis (Dewulf et al. 1995; Jones et al. 1991; Lyons et al. 1989).

TGF-β and BMP7 also coadjuvantly stimulate angiogenesis in the chick chorio-allantoic membrane assay (Ramoshebi and Ripamonti 2000), and collaborately activate prostate cancer cells (Buijs et al. 2007) and osteoblast differentiation. In contrast, BMP7 counteracts TGF-β-induced EndMT in a model of cardiac fibrosis, rendering the EC capable of preserving their endothelial identity (Zeisberg et al. 2007), suggesting a context dependency of the synergy between TGF-βs and BMPs.

The numerous BMP ligands and type I receptors exert a variety of effects on ECs, yet the fact that different ligands utilize common pathway components raises important questions, which may have been neglected until recently: how cells respond specifically to individual ligands, and how cells integrate and interpret signals received from multiple ligands? Concerning this context, worthy of note are studies suggesting that preformed BMP receptor complexes or BMP-induced oligomerization of type I and type II receptors predominantly activate Smad-dependent and -independent signaling, respectively (Nohe et al. 2002; 2004). Also, the choice of type II receptor can persuade the signaling outcome of BMP stimulation as downstream-specific binding of Limk1 to the BMP type II receptor, but not to TGF-β or activin type II receptors (Foletta et al. 2003; Lee-Hoeflich et al. 2004). More recently, different R-Smad complex formation, Smad1/5–Smad2 versus Smad1/5–Smad3, was described (Gronroos et al. 2012), opening the possibility that the novel complexes may be the source of antagonistic versus synergistic responses in different studies.

Evidently, signaling through non-Smad effectors, the recruitment of distinct type II receptors and perhaps more importantly the variability created by alternative Smad complex formation should be further examined as the explanations for the diverse effects.

14.2 Physiological Role of the TGF-β Family in the Vasculature

14.2.1 Physiological Role of ALK1 in the Vasculature

The importance of this receptor became obvious, when Alk1 loss of function studies revealed that its complete loss causes embryonic lethality at midgestation, due to

severe vascular abnormalities, which included vessels hyperdilation, AVMs resulting from fusion of major arteries and veins, and impaired recruitment of VSMC (Oh et al. 2000; Urness et al. 2000). Mutations in the Alk1 gene have been identified as an underlying cause for development of HHT, a rare, human autosomal dominant disease characterized by the presence of recurrent epistaxis and small characteristic malformations of the peripheral blood vessels near the surface of the skin or mucosal linings (Geirdal et al. 2012). AVMs of the lung, liver, and central nervous system are also known clinical findings. Interestingly, EC-specific deletion of the Alk1 gene in the mouse results in neonatal lethality at P5, with the pups exhibiting hemorrhaging brain, lung, and gastrointestinal tract (Park et al. 2008). In attempts to evaluate the contribution of ALK1 to vascular homeostasis in adult mice, Park et al. (2009) deleted the Alk1 gene by tamoxifen administration in 2 months old $R26^{+/CreER}$ $Acvrl1^{2loxP/loxP}$ mice. Tamoxifen-induced Alk1 deletion resulted in severe internal hemorrhage in lung, small intestine, and uterine vessels, and ultimately fatality.

Strong expression of ALK1 has been reported during developmental and neonatal stages, while suppressed during adulthood, except in certain organs, e.g., the lungs (Park et al. 2008). Supportive of that is the observation that ALK1 is fundamental for umbilical and placental blood vessel formation (Hong et al. 2007). However, ALK1 expression is induced in feeding arteries and newly formed blood vessels during wound healing, in adult subdermal blood vessels (Park et al. 2009). AVMs appearing only in subdermal blood vessels where a wound was inflicted provides in vivo experimental evidence that genetic predisposition by endoglin or ALK1 mutations is not enough for development of de novo AVMs in HHT (Park et al. 2008). Interestingly, only selected vascular beds in HHT patients develop telangiectasias or AVM lesions, while other areas (>99.9 %) remain normal (Sadick et al. 2006).

Inhibition of ALK1 by systemic injection of an ALK1 soluble extracellular domain efficiently impaired retinal neonatal angiogenesis, described and validated in three independent studies (Larrivee et al. 2012; Niessen et al. 2010; Ricard et al. 2012). These data implied that ALK1 signaling blockade induced retinal hypervascularization and appearance of AVMs in neonatal mice. Incidentally, the most recent studies also report a cooperative effect of ALK1 and Notch signaling pathways (Larrivee et al. 2012; Morikawa et al. 2011; Ricard et al. 2012). The reported synergy between ALK1 and Notch pathways generated exacerbation of the hypervascularization phenotype, inducing potentiated expression of Notch target genes in the stalk cells, which concomitantly suppress VEGF signaling to the endothelial tip cell (Larrivee et al. 2012). In parallel studies, endothelial-specific inactivation of Smad1/5 in mouse embryonic development yields impaired Dll4/Notch signaling and augmented tip cell in detriment to stalk cell number (Moya et al. 2012). These studies put forward a regulatory crosstalk loop among BMP9/ALK1/Smad1/5 and Notch signaling coordinating tip versus stalk cell specification.

Additionally to being expressed by blood ECs, ALK1 is also expressed by lymphatic ECs (Niessen et al. 2010). In vitro stimulation of lymphatic ECs by BMP9 generates downstream target gene transactivation. Furthermore, inhibition of ALK1

signaling by means of an ALK1-Fc soluble fusion protein diminishes neonatal retinal lymphangiogenesis, while the use of ALK1 targeting monoclonal antibody also impairs Lyve-1 positive lymphangiogenesis in mammary fat pad–implanted MDA-MB-231 breast carcinoma xenografts (Hu-Lowe et al. 2011). Of note, lymph vessel development comprises coordinate and synergistic ALK1 and VEGFR3 signaling regulation, evocative of the crosstalk observed between ALK1/ALK5, ALK1/ALK2, and VEGF-receptor signaling in blood vessel angiogenesis (Cunha et al. 2010; Mitchell et al. 2010).

14.2.2 Physiological Role of Endoglin in the Vasculature

Endoglin, an auxiliary receptor for TGF-β, is required for angiogenesis during development (Li et al. 1999). It is expressed primarily in ECs and its expression is substantially incremented during EC activation, inflammation, ischemia, and tumor angiogenesis (Docherty et al. 2006; Jonker and Arthur 2002; Torsney et al. 2002). The mechanisms involved in endoglin upregulation are presumably multifactorial, but hypoxia is a probable inducer as it prevails in most pathophysiological environments where endoglin is enhanced (Bernabeu et al. 2009).

Endoglin associates with type II receptors of the TGF-β family in the presence of ligand and with the type I signaling receptors, ALK1 and ALK5, even in the absence of exogenous ligand (Barbara et al. 1999). Despite possessing no enzymatic activity, endoglin has been reported to be necessary to modulate ligand-receptor interaction in ALK1, but not in ALK5 signaling (Cheifetz et al. 1992; Lebrin et al. 2004; Pece-Barbara et al. 2005; Scharpfenecker et al. 2007). More recently, ALK5 was shown to phosphorylate the cytoplasmic domain of endoglin in ECs (Ray et al. 2010). Depending on the serine phosphorylation status, only on 646 or on both 646 and 649 serine residues, results in loss of endoglin-mediated inhibition or activation of Smad1/5/8 signaling, respectively, in response to TGF-β/BMP9 signaling (Ray et al. 2010). Taken together, these results indicate that endoglin phosphorylation by ALK5 is an important mechanism for regulating TGF-β and BMP signaling in ECs.

Even though endoglin has an undeniably well-documented connection to ALK1 and its signaling enhancement (Blanco et al. 2005; Lebrin et al. 2004), it is imperative to mention that it interacts not only with other ligands than TGF-β and BMP9 (TGF-β3, activin A, BMP2, and BMP7) but also with several different type I and type II receptors involved in BMP and TGF-β signaling (Barbara et al. 1999). Alternatively, endoglin has also been implicated in interactions with cytoplasmic proteins such as Zyxin, ZRP-1, β-Arrestin, and Tctex2β, which may further generate additional cellular outcomes (Bernabeu et al. 2009). The signaling unrestrictiveness of endoglin consequently adds an extra degree of intricacy to the elaborated signaling networks deriving from the TGF-β family in the angiogenesis field. Moreover, the fact that endoglin is positively associated with ECs proliferation, while weakly expressed in quiescent endothelium, has focused the interest on endoglin as a potential target for cancer in vivo.

14.2.3 Physiological Role of TGF-β in the Vasculature

TGF-β effect in vascular biology is traditionally regarded as an accessory pathway that primarily participates in VMCs differentiation and maturation. However, apart from the genetic evidence for TGF-β relevant direct role in the ECs, there is additional in vitro evidence revealing that several key angiogenic mediators such as VEGF and connective tissue growth factor are direct targets of the TGF-β signaling pathway (Padua and Massague 2009; Sanchez-Elsner et al. 2001). Interestingly, hypoxic conditions present at the core of a tumor juxtaposed to TGF-β signaling can induce robust levels of VEGF mRNA through the activation of hypoxia-inducible factor (HIF)-1 and Smad proteins (Sanchez-Elsner et al. 2001). TGF-β can also regulate the expression, secretion, and activity of matrix metalloproteinase (MMP)-2 and MMP-9, and downregulate the expression of the protease inhibitor TIMP in the tumor and ECs (Derynck et al. 2001; Hagedorn et al. 2001). This TGF-β-mediated metalloprotease activity can subsequently enhance the migratory and invasive properties of ECs required for angiogenesis.

Multiple genetic mouse models suggestive of a direct role for TGF-β signaling pathway in angiogenesis further corroborate the need to more thoroughly analyze this pathway in the tumor context. Increased expression of TGF-β in either prostate carcinoma cells or Chinese hamster ovary cells resulted in robust angiogenic responses, which could be blocked by TGF-β neutralizing antibodies (Tuxhorn et al. 2002; Ueki et al. 1992). In sharp contrast, LNCaP human prostate xenografts treated with TGF-β neutralizing antibodies exhibited a 2- to 3.5-fold reduction in microvessel density vessels relative to control animals, whereas blood lakes were found in some areas of the tumors (Tuxhorn et al. 2002). Additionally, a TGF-β small molecule inhibitor LY364947 impaired tumor angiogenesis and growth in a transgenic multistep pancreatic islet tumor model (Cunha, SI, unpublished observation; Fig. 14.3). Another study using the orthotopic xenograft model of breast cancer MDA-MB-231 reported that ALK5-mediated TGF-β signaling is critical for metastasis dissemination and tumor angiogenesis in MMP9-regulated fashion (Safina et al. 2007). In parallel, Smad2 and Smad3 were reported to have opposing roles in breast cancer bone metastasis by differentially affecting VEGF-mediated tumor angiogenesis in an experimental mouse model of bone metastasis, where osteotropic MDA-MB-231-luc cells were intracardially inoculated (Petersen et al. 2010).

To sum up, both direct and indirect effects of TGF-β on the tumor microenvironment stimulate tumor angiogenesis.

TGF-β affects tumor angiogenesis per se but it also regulates vascular permeability. Neutralizing antibodies to TGF-β1 or an ALK5 inhibitor significantly augment vessel permeability. In two distinct transgenic mouse tumor models, MMTV-PyMT and K14-HPV16, breast and epidermal squamous cell cancers, respectively, inhibition of ALK5 further enhanced vascular leakage into the interstitium and facilitated increased delivery of high molecular weight compounds into premalignant and malignant tissue (Lammerts et al. 2002; Sounni et al. 2010). These data strongly suggest that ALK5 antagonists can be therapeutically exploited to improve the

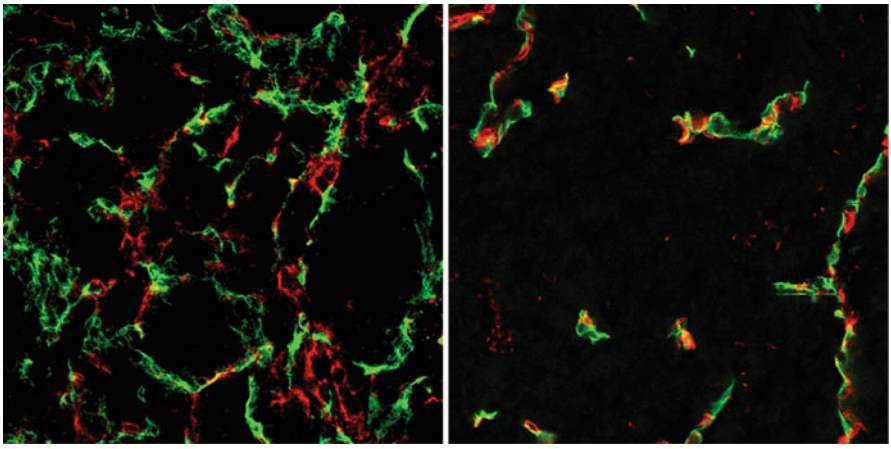

Fig. 14.3 Inhibition of ALK5 signaling by the small molecule LY364947 inhibits tumor angiogenesis in the RIP1-Tag2 mouse model of pancreatic neuroendocrine cancer (immunostaining for the endothelial cell marker podocalyxin, *green* and the pericyte marker NG2, *red*)

delivery of drugs or molecular contrast agents into tissues where chronic damage or neoplastic disease limits their efficient delivery. Along with these observations, another study compared the effects of two different tyrosine kinase inhibitors, imatinib and sorafenib, with an ALK5 inhibitor (LY364947) on extravasation of a modeled nanoparticle, 2 MDa dextran in two tumor models: the CT26 colon cancer model and the BxPC3 pancreatic cancer model (Kano et al. 2009). In fact, sorafenib most potently enhanced the accumulation of nanoparticles in the CT26 colon cancer model, whereas TGF-β inhibitor exhibited a stronger effect on the BxPC3 pancreatic cancer model, suggesting that while ALK5 inhibitors are an appropriate strategy to enhance delivery of nanoparticle-delivered drugs in pancreatic tumors, this may not be longitudinally optimal for all tumor types.

A small molecule ALK5 inhibitor at a low dose was used for treating several experimental intractable solid tumors, including pancreatic adenocarcinoma and diffuse-type gastric cancer, characterized by hypovascularity and thick fibrotic tumor stroma. Low doses of ALK5 inhibitor altered TGF-β signaling neither in the malignant cells nor in the cancer-associated fibroblast components. However, the ALK5 inhibitor decreased pericyte coverage on the endothelium without reducing endothelial area specifically in the tumor vasculature. As a result, ALK5 inhibition promoted accumulation of anticancer nanocarriers in the tumors. In the absence of ALK5 inhibitor, the anticancer nanocarriers exhibited poor growth-inhibitory effects (Kano et al. 2007).

The vasculature of solid tumors is abnormal, both in terms of vessel architecture and blood flow dynamics. Permeable heterogeneous vessel walls permit the leakage of proteins and fluid that, coupled with inefficient lymphatic drainage, hinder drug delivery. As acute blockade of TGF-β signaling transiently alters vessel stability,

permeabilization of tumor vascular beds improves intravenous delivery of high molecular weight compounds to the tumor such as antibodies or nanoparticles that are therapeutically selective, collectively rendering an improved clinical outcome. This effect has particular relevance when treating stroma-rich intractable solid tumors. Furthermore, by promotion of a more pervious tumor vessel phenotype, not only the delivery of standard therapeutic agents can be improved but also diagnostic molecular imaging agents can be more easily monitored in tumor tissue where TGF-β signaling has been transiently impaired.

Most studies on the role of TGF-β in cancer primarily rely on the effect of this cytokine on the cancerous epithelial cells and on how it affects cellular plasticity, more specifically epithelial-to-mesenchymal transition (EMT) and metastatic dissemination. Here, we focus essentially on the effects of TGF-β signaling on the tumor vasculature; however some of the studies on metastasis may have affected TGF-β-mediated vasculature leniency to tumor cells intra- and extravasation. In fact, some laboratories have shown that the metastatic spread of the 4 T1 breast cancer transplantable model of metastatic breast cancer can be efficiently suppressed by administering an antibody that targets all three isoforms of the TGF-β ligand. This work went on to show that TGF-β neutralizing antibodies have multiple cooperative effects on angiogenesis, immune cell function, and tumor cell viability, eventually leading to effective tumor growth control and reduction of metastatic foci (Nam et al. 2008).

14.3 Perspective

The formation of new blood vessels in pathological processes, such as cancer, leads to unstable, proliferative suboptimally functional vessels in a dynamic state of remodeling. Tumor vascular networks support tumor growth, in spite of a range of abnormal features that compromise their physiology. Tumor vessels are commonly tortuous and leaky, causing hemorrhage and increased interstitial fluid pressure (Carmeliet and Jain 2000). Inefficient blood flow caused by poor hierarchical anatomy and organization of the tumor vasculature leads to ischemia and necrosis, which are common characteristics of rapidly growing tumors.

Its inherent instability should make tumor vascular beds distinct from normal vessels and therefore more prone to be exquisitely affected by selective targeting anti-angiogenic drugs (Baluk et al. 2005). During the past few years we have witnessed a suboptimal outcome from anti-angiogenic treatment of human cancer implying the need for improvement and suggesting that alternative therapeutic avenues should be explored. Better knowledge of molecular and cellular mechanisms taking place in the tumor microenvironment and in the tumor vasculature is essential to achieve such goals.

The complexity of the TGF-β and BMP pathways by means of redundancy, cooperation, or by simply having different levels of regulation reflects the uniqueness and tight tissue specificity of this intriguing pathway. However, despite being

complex, the TGF-β signaling pathway may hold central roles in tumor angiogenesis, as suggested by studies targeting its endothelial-specific receptors ALK1 and endoglin. Inhibitors for these receptors have already been generated and they hold great promise in impairment of de novo formed tumor vessels, while leaving normal idle vasculature undisturbed. The preclinical development and clinical use of inhibitors of pro-angiogenic TGF-β signaling will be discussed in detail in Chap. 19.

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Chapter 15
TGF-β Signaling Pathway and MicroRNAs in Cardiovascular Disease

Nisha Marathe and Akiko Hata

Abstract Transforming growth factor-β (TGF-β) signaling has long been known to influence development and function of different organs, including the cardiovascular system. In addition to the canonical pathways of Smad-dependent transcriptional regulation of protein-coding genes, recent studies have shown TGF-β influence on microRNA (miRNA) expression to play a critical role in TGF-β-dependent biological effects. A number of miRNAs, such as miR-143, miR-145, miR-21, miR-155, and miR-26a, are identified as critical downstream mediators of the TGF-β-family signaling. Deregulation of miRNA expression can result in various developmental defects and diseases. In this review, we summarize the latest view of the effects of miRNAs associated with TGF-β-family signaling on the cardiovascular system.

Keywords Bone morphogenetic protein • MicroRNA • Precursor microRNA • Smad-primary microRNA • Transforming growth factor-β • Vascular smooth muscle cell

Abbreviations

BMP	Bone morphogenetic protein
miRNA	MicroRNA
pre-miRNA	Precursor microRNA
pri-miRNA	Primary microRNA
TGF-β	Transforming growth factor-β
VSMC	Vascular smooth muscle cell

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15.1 Introduction

The TGF-β superfamily of growth factors, including TGF-βs and bone morphogenetic proteins (BMPs), is involved in multiple cellular processes such as cell differentiation, proliferation, and apoptosis (Bobik 2006). Aberrant gene expression in vascular smooth muscle cells (VSMC), endothelial cells, fibroblasts, and cardiac myocytes—key cellular components of the cardiovascular system—contributes to heart attack, stroke, peripheral vascular disease, hypertension as well as congenital heart defects (Toma and McCaffrey 2012). Both TGF-β and BMP signaling are required for healthy cardiac development and function (Bobik 2006). TGF-β acts upon ligand binding to the TGF-β receptor type-II (TGF-βRII) which then recruits and phosphorylates the TGF-β receptor type-I (TGF-βRI) to form a type I/type II receptor complex. Activation of the BMP receptor heterodimer is accomplished in a similar manner (Shi and Massague 2003). The receptor complex then phosphorylates a receptor-specific signal transducer known as R-Smad—the TGF-β receptor complex transmits a signal through Smad 2 and 3, while Smad 1, 5, and 8 are responsible for the BMP signaling. Upon phosphorylation by the type I receptor kinase, R-Smads form a complex with the common-Smad (co-Smad), Smad4, and translocate into the nucleus where the R-Smad/co-Smad complex binds to the DNA sequence known as the Smad binding element (SBE) together with cofactors which associate with the sequence adjacent to the SBE, and modulates transcription positively or negatively (Butz et al. 2012) (Fig. 15.1). More recently, R-Smads are shown to regulate miRNA biogenesis at the processing step in the nucleus and modulate their expression (Davis et al. 2008) (Fig. 15.3).

MiRNAs are small (20–23 nucleotides, nt) noncoding RNAs involved in post-translational regulation of gene expression (Blahna and Hata 2012). MiRNAs typically bind to the partially complementary sequence in the 3′-untranslated region (3′-UTR) of messenger RNAs (mRNAs) and negatively regulate their expression through either translational inhibition or promotion of mRNA degradation (Filipowicz et al. 2008). Genome-wide analyses of 3′-UTRs suggest more than 60 % of mRNAs can be targeted by miRNAs (Friedman et al. 2009). Thus, miRNAs are important modulators of protein expression. Of the 20–23 nt of the mature miRNA sequence, a 6–8 nt sequence at the 5′ end, known as the "seed sequence," is usually sufficient to confer target specificity, although under certain conditions other nucleotides of the mature miRNA sequence are also important (Grimson et al. 2007). Regions in the 3′-UTR complementary to the miRNA seed region are known as "miRNA recognition element (MRE)" and result in downregulation of the gene in the presence of corresponding miRNAs. Due to the relatively short miRNA recognition sequence, a large number of genes can be targeted by a single miRNA; conversely, a single mRNA can be targeted by multiple miRNAs.

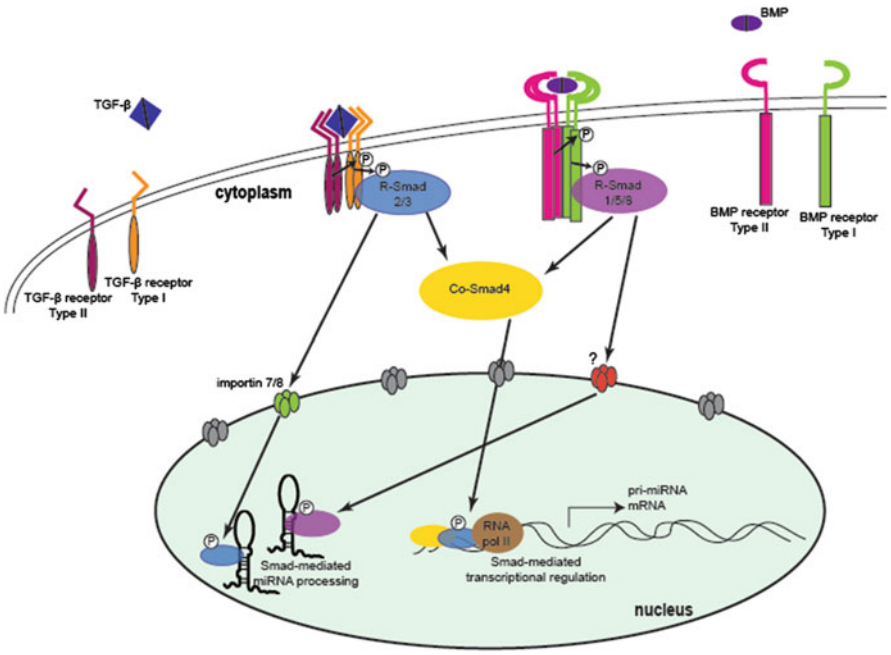

Fig. 15.1 Schematic representation of TGF-β/ BMP signaling. TGF-β/ BMP bind to the Type II receptor which recruits and transphosphorylates the Type I receptor. Phosphorylation of R-Smads by the activated Type I receptor kinase induces association with Co-Smad4 and translocation into the nucleus to regulate transcription. Alternately, R-Smads can regulate miRNA biogenesis in the nucleus. Smad2/3 are imported to the nucleus via importins 7 and 8. The mechanism of nuclear import of R-Smad1/5/8 is yet to be characterized

15.1.1 MiRNA Biogenesis

MiRNAs are synthesized as a long primary transcript (pri-miRNA) by RNA polymerase II. Pri-miRNAs contain a 5′-7-methyl-guanosine cap and 3′-polyadenylation similar to mRNAs (Fig. 15.2). A majority of miRNA genes are encoded in intergenic regions in the DNA and are transcribed independently; however, 30–50 % of miRNAs lie within the introns and are transcribed and regulated in conjunction with the host gene (Rodriguez et al. 2004). The pri-miRNA is subsequently processed in the nucleus by the Drosha complex whose core components include an RNase III enzyme, Drosha, and a cofactor, DiGeorge critical region 8 (DGCR8) (Lee et al. 2003; Gregory et al. 2004) (Fig. 15.2). Other proteins associated with the Drosha complex include RNA helicases, such as p68 and p72 (Fukuda et al. 2007). Drosha cleaves the pri-miRNA into a shorter precursor-miRNA (pre-miRNA). DGCR8 is a double-stranded RNA binding protein and stabilizes the interaction between Drosha and the pri-miRNA (Yeom et al. 2006). p68 (also known as DDX5) and p72

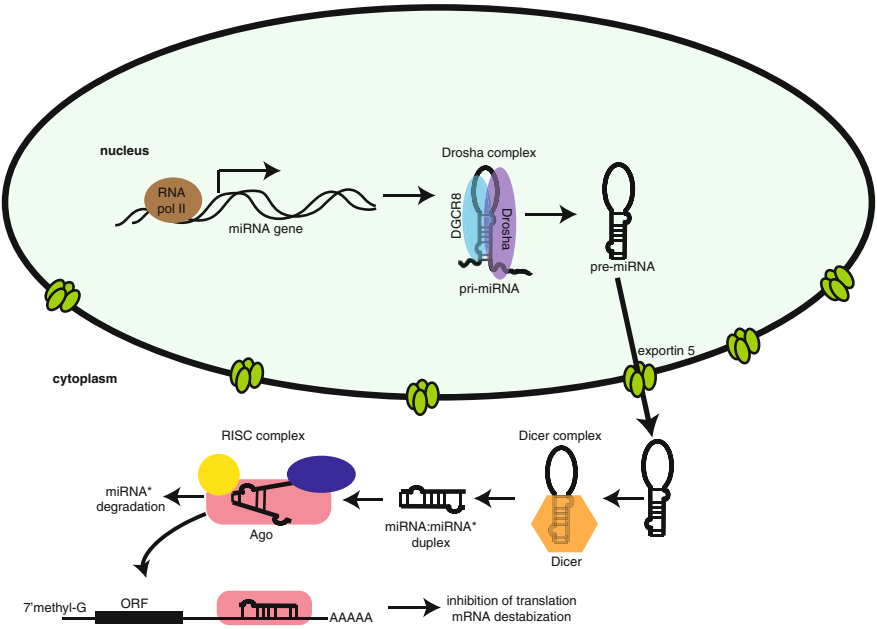

Fig. 15.2 MiRNA biogenesis pathway. MiRNA is transcribed into a long primary transcript known as pri-miRNA by RNA pol II. Pri-miRNA is then cleaved by the Drosha complex, which is composed of Drosha, DGCR8, and other cofactors to generate precursor miRNA (pre-miRNA). Pre-miRNA is then exported from the nucleus through exportin 5. In the cytoplasm, the pre-miRNA is cleaved by the Dicer complex to generate a mature miRNA duplex. The miRNA duplex is then loaded onto the RISC in which one of the two strands (known as the guide strand) is selected for target gene silencing. The other strand (known as the passenger strand) is rapidly degraded

(also known as DDX17) are both DEAD-box RNA helicases and the helicase activity is essential for miRNA processing (Salzman et al. 2007). p68 and p72 each is known to control processing of distinct, but overlapping, subsets of miRNAs (Fukuda et al. 2007).

The pre-miRNA is composed of a stem-loop structure with the mature miRNA sequence encoded in the stem region (Davis and Hata 2009) (Fig. 15.3 shown in red line). Following cleavage by the Drosha complex, the pre-miRNA is exported to the cytoplasm through exportin-5 and further processed into the mature miRNA by the cytoplasmic RNase III enzyme called Dicer (Fig. 15.2). Dicer recognizes the stem-loop structure of the pre-miRNA and cleaves the pre-miRNA into a ~22 bp RNA duplex. Argonaut proteins (Ago1-4 in human) associate with the miRNA duplex, separate the two strands, and choose one of the strands, which is called the "guide strand," and present it to the RNA-induced silencing complex (RISC), which then targets and silences the mRNA (Davis and Hata 2009). The strand that is not selected by Argonaut is known as the "passenger strand" and is rapidly degraded. In some cases, both the guide and passenger strands actively participate in mRNA silencing (Fig. 15.2).

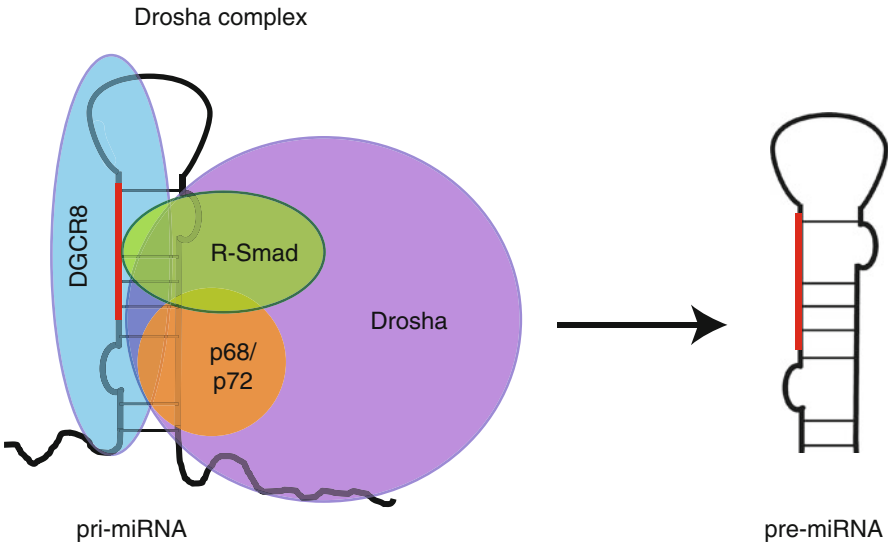

Fig. 15.3 MiRNA processing by Smads. Processing of a subset of pri-miRNAs is mediated by the Drosha complex in a p68 and R-Smad-dependent manner. R-Smads facilitate the recruitment of Drosha and promote the cleavage of pri-miRNAs by Drosha. The red line in the stem region of the pri-miRNA indicates a sequence encoding mature miRNA

In contrast with transcriptional regulation of genes, which can mediate dramatic changes, miRNAs often effect subtle changes in gene expression. MiRNA-mediated gene silencing typically reduces protein and/or mRNA expression by ~30–50 % (Selbach et al. 2008). Despite the relatively small effect, miRNAs are critical for development and the maintenance of homeostasis (Selbach et al. 2008; Baek et al. 2008; Hata and Davis 2010). Indeed, mice deficient in DGCR8, p68, p72, Dicer, or Ago2 die early in gestation due to gross developmental defects (Wang et al. 2007; Fukuda et al. 2007; Bernstein et al. 2003; Morita et al. 2007). MiRNA expression can be controlled at the transcriptional level, as well as at either cropping step by Drosha or Dicer (Bauersachs and Thum 2011; Davis et al. 2008; Adams et al. 2009). This allows for fine-tuning of miRNA expression and, subsequently, silencing of the miRNA target genes. In this review, we summarize the functional relationship between the TGF-β signaling pathway and miRNAs.

15.1.2 Transcriptional Regulation of miRNAs by TGF-β

TGF-β is known to regulate transcription via the R-Smad/co-Smad complex (Shi and Massague 2003) (Fig. 15.1). TGF-β2 controls endothelial-to-mesenchymal transition (EndMT) of mouse endothelial cells isolated from heart microvasculature.

In response to TGF-β2 treatment, cells become more fibroblast-like and miRNAs associated with fibroblasts are induced (Ghosh et al. 2012). Interestingly, deregulation of these miRNAs, including miR-125b, miR-21, miR-30b, miR-195, and miR-375, is often associated with cardiovascular diseases, such as hypertrophy, cardiac fibrosis, arrhythmia, myocardial infarction, heart failure, and cardiomyopathy (Ghosh et al. 2012). The same rules of transcriptional regulation by the Smad complex that apply to protein coding genes can be applied to the transcriptional regulation of miRNAs (Ozsolak et al. 2008; Corcoran et al. 2009; Chung et al. 2010). During canonical TGF-β signaling, the inhibitory Smad, Smad7, competes with R-Smads for binding at the type I TGF-β receptor to decrease R-Smad phosphorylation and signaling; similarly, Smad7 can also repress miRNA expression. For example, Smad3-dependent miR-192 expression is reduced by upregulation of Smad7 in rat tubular epithelial cells (Chung et al. 2010). Furthermore, Smad4 seems to be a key regulator of transcriptionally Smad-dependent miRNAs. TGF-β stimulation of Smad4-deficient murine mammary gland epithelial cells resulted in deregulation of 28 miRNAs (Kong et al. 2008). Since Smad4 is required upon ligand stimulation for the nuclear translocation of R-Smads and binding to the SBE and is involved in both TGF-β and BMP signaling, it is not surprising that Smad4 is critical for transcriptional control of miRNAs by TGF-β and BMP signaling.

15.1.3 Posttranscriptional Regulation of miRNAs by TGF-β

In addition to transcriptional regulation, miRNAs are also subject to regulation during maturation—namely, at the cleavage of pri-miRNAs to generate pre-miRNAs by the Drosha complex in the nucleus (Fig. 15.3). In VSMC, TGF-β, and BMP have a critical role in regulating VSMC phenotype by modulating miRNA expression. In response to TGF-β and BMP signaling, R-Smads bind to the stem region in the pri-miR-21 encoding mature miR-21 and facilitate its processing to pre-miR-21 (Davis et al. 2008). Upon BMP or TGF-β stimulation, no change of pri-miR-21 levels is observed; however, an increase in pre-miR-21 levels can be detected, suggesting a posttranscriptional regulation of miR-21 by Smads (Davis et al. 2008). RNA-immunoprecipitation assays place R-Smads in a complex with p68 and Drosha upon TGF-β or BMP stimulation (Davis et al. 2010). Interestingly, TGF-β or BMP stimulation also results in increased Drosha recruitment to pri-miR-21, suggesting that R-Smads may facilitate the recruitment and association of Drosha with specific pri-miRNAs, which in turn leads to more efficient processing of the pri-miR-21 (Davis et al. 2010). This mechanism is proposed to extend to ~20 miRNAs in VSMC (Davis et al. 2010).

Unlike transcriptional regulation, Smad4 does not seem to be required for TGF-β-mediated regulation of miRNA processing, as downregulation of Smad4 by small inhibitory RNA (siRNA) in VSMC does not affect the processing of pri-miR-21 (Davis et al. 2008). Smad4 was previously shown to be required for the nuclear translocation of R-Smads; however, recent studies show that R-Smads and Smad4

can be translocated to the nucleus via an alternate nuclear import machinery known as importin (Imp) 7/8 (Xu et al. 2007; Yao et al. 2008). It can be speculated that the R-Smads that translocate to the nucleus independently from Smad4 are responsible for miRNA processing while those translocating to the nucleus as a complex with Smad4 are involved in transcriptional regulation. The *Arabidopsis* homologue of Smad nuclear interacting protein 1 (SNIP1), DAWDLE (DDL), plays a role in the expression of both pri-miRNAs and pre-miRNAs through transcriptional control. It is proposed that DDL enables access or recognition of pri-miRNAs by Dicer-like 1 (DCL1), the *Arabidopsis* homologue of Dicer (Yu et al. 2008). Since SNIP1 was originally identified as a Smad-interacting protein, it is plausible that SNIP1 promotes miRNA biogenesis through association with Smads.

Interestingly, the mechanism of regulation of miRNA processing in the nucleus is not limited to R-Smads. Other transcription factors, such as estrogen receptor-α (ER-α) and p53, have been reported to regulate the catalytic activity of Drosha (Suzuki et al. 2009; Yamagata et al. 2009). Upon activation of ER-α or p53, these proteins interact with the Drosha complex through association with p68 and modulate processing of a distinct set of miRNAs (Yamagata et al. 2009; Suzuki et al. 2009).

15.1.4 Regulation of TGF-β Signaling by miRNAs

Smads themselves are subject to regulation by miRNAs. The transcripts encoding Smad proteins have been identified as targets of miRNA(s) (Blahna and Hata 2012). This introduces an additional level of complexity to the regulation of miRNAs by enabling positive- or negative-feedback loops. For example, Smad5 is a target of miR-135 (Li et al. 2008). Upon BMP2 stimulation in murine pre-myoblast cells, miR-135 is downregulated, enabling elevated expression of Smad5 and increased BMP signaling (Li et al. 2008). Conversely, miR-26a forms a negative-feedback loop with Smad1. In human adipose-derived stem cells, miR-26a expression is increased during differentiation thereby limiting the duration of Smad signaling by targeting Smad1 (Luzi et al. 2008). In addition to Smads, other proteins involved in TGF-β or BMP signal transduction are also known to be targeted by miRNAs. These include ZEB2 (also known as Smad interacting protein 1, SIP1) (Kato et al. 2007; Hu et al. 2010), the TGF-β receptor type II (TGF-βRII) (Kim et al. 2009), the BMP receptor type II (BMPRII) (Qin et al. 2009), and Smad7 (Liu et al. 2010). The TGF-β2 ligand is also targeted by miR-200a (Wang et al. 2011).

15.1.5 TGF-β and Cardiovascular Disease

Pathways activated by TGF-β family ligands have been implicated in a number of cardiovascular diseases. Elevated TGF-β expression leads to transcriptional activation of connective tissue growth factor (CTGF) implicated in the development of

atherosclerotic plaques (Ohyama et al. 2012). CTGF is a key component of the extracellular matrix (ECM) and promotes a pathological phenotype by inducing ECM deposit and monocyte infiltration (Ohyama et al. 2012). BMP also leads to vascular inflammation and calcification resulting in the formation of atherosclerotic plaques (Derwall et al. 2012). BMPs can promote differentiation of multipotent cells to the osteoblastic lineage, thereby increasing vascular calcification (Cai et al. 2012). Therefore, negative control of TGF-β family signaling is crucial for preventing the formation of atherosclerotic lesions.

TGF-β is also a key effector of myocardial fibrosis. In a pressure-overload model, activation of fibroblasts leads to myocardial fibrosis, which can be alleviated by treatment with a neutralizing antibody against TGF-β (Kuwahara et al. 2002). TGF-β also induces cells to undergo EndMT. Aberrant TGF-β signaling can lead to cardiac fibrosis due to the influx of mesenchymal cells in the heart (Kovacic et al. 2012). It was also recently reported that TGF-β-induced activation of Rho-associated kinase (ROCK) leads to suppression of BMP signaling and increases fibrosis (Wang et al. 2012d). In addition, angiotensin II-induced IL-6 increases TGF-β expression and signaling, leading to cardiac fibrosis (Ma et al. 2012).

15.2 TGF-β Regulates VSMC Phenotype

Arterial hypertension is another pathological condition associated with abnormal TGF-β signaling. In patients, increased TGF-β expression correlates with increased arterial pressure. Increased TGF-β signaling in Emilin1 knockout animals causes hypertension, which can be controlled with genetic ablation of TGF-β1 (Gordon and Blobe 2008). TGF-β signaling is suppressed by hypoxia-inducible factor 1 (HIF-1) to prevent myocardial hypertrophy in a transaortic constriction mouse model (Wei et al. 2012). Additionally, pulmonary artery hypertension (PAH) is highly associated with mutations in the gene encoding BMPRII (Machado et al. 2006; Santibanez et al. 2011). About 70–80 % of heritable PAH and 15–40 % of idiopathic PAH are associated with a BMPRII mutation (Machado et al. 2006; Thomson et al. 2000). It has been suggested that a number of the nonsense or missense mutations identified in *BMPRII* produce mRNA transcripts which would be susceptible to nonsense-mediated mRNA decay, or inactivation of BMP receptor function as a result of (a) accumulation in the endoplasmic reticulum and reduced expression on the cell surface, (b) lack of ligand binding, or (c) inactivation of serine/threonine kinase activity (Morrell 2010), which in turn cause downregulation of the BMP signal transduction.

VSMC compose the medial layer of the blood vessel wall and are critical for contraction and relaxation of the vessel to control blood pressure. Although differentiated, VSMC are plastic and undergo a phenotype switch in response to cellular stimuli or injury—the differentiated "contractile" state is characterized by a low proliferation rate, expression of smooth muscle cell (SMC)-specific contractile genes, and the capacity to contract upon stimulation, while the dedifferentiated

"synthetic" state exhibits greater proliferation, migration, and reduced expression of contractile genes (Owens et al. 2004). This phenotype switching allows VSMC to repair injuries by facilitating replacement of damaged cells; however, prolonged time in the synthetic state can lead to progression of vascular proliferative diseases, such as atherosclerosis, PAH, or restenosis after angioplasty (Owens et al. 2004). TGF-β and BMP promote VSMC towards the contractile phenotype exhibited by an elongated, spindle-like morphology, inhibition of proliferation and migration, and the expression of the SMC-specific contractile genes α-actin (α-SMA), SM22α, and calponin (Rensen et al. 2007).

Recent studies have implicated the involvement of multiple miRNAs in the control of the VSMC phenotype (Davis et al. 2009; Liu et al. 2009; Cheng et al. 2009; Kang and Hata 2012). Mice deficient in Dicer in SMC result in embryonic lethality with severe defects in the vessel wall structure and in the medial layer of the vessel, indicating the critical role of miRNA in SMC during embryogenesis (Albinsson et al. 2010). Additionally, mice carrying an inducible SMC-specific Dicer deletion display a loss of contractile function in their umbilical arteries (Albinsson et al. 2010). MiRNAs play a key role in the development and function of SMC in association with TGF-β signaling. In the following section, we review individual miRNAs downstream of TGF-β or BMP signaling which play important roles in cardiovascular biology.

15.3 MiRNAs Regulated by TGF-β in Cardiovascular Tissues

15.3.1 MiR-143/145

MiR-143 and miR-145 are exclusively expressed in VSMCs and are directly and indirectly regulated transcriptionally by TGF-β and BMP (Long and Miano 2011; Davis-Dusenbery et al. 2011). Activation of Smads by TGF-β and BMP leads to upregulation of myocardin and myocardin-related transcription factors (MRTFs), respectively. These factors form a complex with the serum response factor (SRF) and induce transcription of miR-143 and miR-145 through the DNA sequence known as the CArG box (Cordes et al. 2009; Xin et al. 2009). Both miR-143 and miR-145 are originally transcribed as a single pri-miRNA which is processed into two distinct miRNAs (Iio et al. 2010). Both miR-143 and miR-145 promote the contractile phenotype in VSMCs by allowing CArG box-dependent contractile gene expression and inhibiting VSMC proliferation. Among the targets of miR-143 and miR-145 are Kruppel-like factor 4 (KLF4), Kruppel-like factor 5 (KLF5), and E twenty-six (ETS)-like transcription factor 1 (ELK1), which promote the synthetic phenotype (Davis-Dusenbery et al. 2011; Rangrez et al. 2011). Knockdown of miR-143/145 in SMC inhibits differentiation of VSMC and prevents contraction in the presence of the appropriate cellular stimuli (Davis-Dusenbery et al. 2011; Long and

Miano 2011). MiR-143/145 knockout mice exhibit a reduced medial layer, a general decrease in all contractile gene expression and disorganized actin stress fibers in VSMC (Xin et al. 2009; Boettger et al. 2009; Elia et al. 2009). Additionally, mice exposed to chronic hypoxia develop PAH and express high levels of miR-145 in the smooth muscle layer of vessels, whereas miR-145 knockout mice are protected from PAH (Caruso et al. 2012). PAH patients with *BMPRII* mutations also exhibit high levels of miR-145 in SMC (Caruso et al. 2012).

15.3.2 MiR-21

MiR-21 expression showcases the posttranscriptional mode of regulation of miRNAs by TGF-β and BMP. Upon TGF-β and BMP stimulation, Smads associate with Drosha and facilitate the processing from pri-miR-21 to pre-miR-21, while no observable increase is detected in the pri-miR-21 (Davis et al. 2008). One of the functional targets of miR-21in VSMC is programmed cell death protein 4 (PDCD4), which promotes the contractile gene expression in VSMC (Davis et al. 2008). MiR-21 is also controlled by Fos-related antigen 1 (Fra-1), which is a target of miR-143 (Horita et al. 2011). Therefore, TGF-β signaling induces SRF, which increases miR-143 expression and decreases Fra-1 expression and controls miR-21. Additionally, miR-21 is known to inhibit the downstream target of Fra-1, phosphatase and tensin homolog (PTEN), creating a unique crosstalk between miR-21-PTEN and miR-143-Fra1 and ultimately reducing cell proliferation. Other targets of miR-21 include Smad7 and Dedicator of cytokinesis (DOCK) 4, 5, and 7, which are known to enhance proliferation and migration of VSMC (Kang et al. 2012; Liu et al. 2010). Hence, induction of miR-21 by TGF-β downregulates VSMC proliferation and migration and promotes the contractile phenotype (Kang et al. 2012).

15.3.3 MiR-29

The miR-29 family of miRNAs (miR-29a/b/c) targets the 3′ UTRs of various collagen proteins including collagen I and IV. The loss of miR-29 has been associated with an increase in pulmonary fibrosis (Xiao et al. 2012). MiR-29 is transcriptionally downregulated by TGF-β (Xiao et al. 2012). Upon TGF-β treatment, inhibition of miR-29 results in elevated expression of the ECM genes (Xiao et al. 2012). Smad3-deficient mice are protected from pulmonary fibrosis due to downregulation of miR-29 (Xiao et al. 2012). Overexpression of miR-29 also suppresses expression of TGF-β1 and CTGF, thereby protecting against bleomycin-induced lung fibrosis (Xiao et al. 2012). Decreased levels of miR-29 are also found in renal fibrosis (Wang et al. 2012a).

15.3.4 MiR-125b

MiR-125b expression is induced transcriptionally by TGF-β in mouse endothelial cells isolated from heart microvasculature which results in increased expression of ECM genes (Ghosh et al. 2012). p53 is a target of miR-125b (Le et al. 2009). p53 functions to regulate the TGF-β-mediated fibrosis by blocking type I collagen synthesis and Smad3-dependent collagen gene transcription by disrupting the association of Smad3 with a transcriptional cofactor, p300 (Ghosh and Vaughan 2012). Thus, increased expression of miR-125b is essential for the progression of fibrosis.

15.3.5 MiR-27b

TGF-β signaling transcriptionally inhibits miR-27b expression (Wang et al. 2012c). Increased levels of miR-27b are found in cardiac myocytes in which Smad4 is deleted leading to cardiac hypertrophy (Wang et al. 2012c). *In vitro* results also confirm a reduction in miR-27b expression upon TGF-β stimulation (Wang et al. 2012c). Conversely, inhibition of miR-27b in vivo using antisense RNA oligonucleotides against miR-27b (antagomir-27b) resulted in less pronounced cardiac dysfunction using a pressure-overload model in mice, indicating a role for miR-27b in cardiovascular disease. Among the validated downstream targets of miR-27b is the peroxisome proliferator-activated receptor γ (PPARγ), a critical modulator of cardiac hypertrophy (Wang et al. 2012c). Activation of PPARγ protects cardiac myoctyes from hypertrophy as measured by cell size and atrial natriuretic factor (ANF) expression (Amin et al. 2010). TGF-β-induced downregulation of miR-27b derepresses the level of PPARγ and protects against cardiac hypertrophy (Wang et al. 2012c). It is of note that TGF-β can also induce cardiac hypertrophy by propagating through Smad-independent signaling involving TGF-β-activated kinase 1 (TAK1) (Xiao and Zhang 2008). Smad signaling is protective against cardiac hypertrophy, revealing the complex nature of TGF-β signaling in cardiomyocytes (Xiao and Zhang 2008; Wang et al. 2005).

15.4 MiRNA Regulation of TGF-β Signaling in Cardiovascular Tissues

15.4.1 MiR-155

MiR-155 targets Smad2, which leads to an inhibition of TGF-β signaling (Kong et al. 2008). Targets of miR-155 include the G-protein coupled receptor, angiotensin receptor (AT1R), involved in the signal transduction of the vasoconstricting

peptide, angiotensin II (Kong et al. 2008). Expression of miR-155 in aortic adventitial fibroblasts leads to the decrease of contractile gene expression and function (Zheng et al. 2010). Interestingly, TGF-β induces expression of miR-155 in epithelial cells through transcriptional activation by Smad4 (Kong et al. 2008). Thus, miR-155 contributes to the ablation of TGF-β signaling through a negative feedback mechanism (Kong et al. 2008).

15.4.2 MiR-26a

It has been demonstrated that miR-26a targets both Smad1 and Smad4, modulating both the BMP and the TGF-β signaling cascades (Leeper et al. 2011). PTEN, which is a validated target of miR-21, is another target of miR-26a (Huse et al. 2009). MiR-26a could be an additional player in the interesting crosstalk between miR-26a/miR-21-PTEN and miR-143-Fra-1 axis. MiR-26a has been shown to be a mechanosensitive miRNA and is upregulated by mechanical stretch in human airway SMC (Mohamed et al. 2010); thus, vascular constriction in hypertension could be attributed to the induction of miR-26a, which in turn downregulates the BMP and/or the TGF-β signaling.

15.4.3 MiR-200

Expression of the miR-200 family of miRNAs, miR-200a/b/c, in alveolar epithelial cells (AEC) prevents TGF-β-induced epithelial–mesenchymal transition (EMT) and induction of pulmonary fibroblasts (Yang et al. 2012). MiR-200 acts by targeting GATA3 and the zinc finger E-box-binding homeobox proteins ZEB1 and ZEB2, critical transcription factors in the TGF-β-mediated EMT. GATA3, ZEB1, and ZEB2 increase transcription of mesenchymal markers, such as Vimentin or N-cadherin while repressing the transcription of epithelial markers, such as E-cadherin (Gemmill et al. 2011). Additionally, miR-200 expression reduces lung fibrosis in both in vitro and in vivo models (Yang et al. 2012). Interestingly, ZEB1 and ZEB2 also suppress expression of the miR-200 family of miRNAs (Gregory et al. 2011), indicating a positive-regulatory loop between ZEB1/ZEB2 and the miR-200 family of miRNAs. When TGF-β signal is activated, AEC cells undergo EMT as a result of TGF-β-dependent repression of the miR-200 family of miRNAs (Gregory et al. 2011).

15.4.4 MiR-24

TGF-β induces miR-24 expression which, in turn, reduces TGF-β secretion and Smad2/3 phosphorylation and activation (Dogar et al. 2011). MiR-24 targets Furin, a protease that converts the latent TGF-β to mature, bioactive TGF-β, in cardiac

fibroblasts; thus, TGF-β signaling is reduced (Wang et al. 2012b). Additionally, when exogenous miR-24 is delivered in vivo, it can reduce cardiac fibrosis. This feedback mechanism provides a novel scheme for TGF-β regulation.

MiR-24 is also induced by platelet-derived growth factor-BB (PDGF-BB), a potent inducer of the synthetic phenotype, and regulates BMP signaling by targeting Tribbles3 (Trb3), a protein which interacts with BMPRII and facilitates BMP signaling via downregulation of Smad ubiquitin-regulatory factor-1 (Smurf1) in VSMC (Chan et al. 2010). Smurf1 negatively regulates BMP signaling by targeting R-Smads (Derynck and Zhang 2003). Therefore, induction of miR-24 by PDGF-BB ultimately downregulates BMP signaling and stimulates proliferation and migration of VSMC (Chan et al. 2010).

15.5 Conclusion

In this review we summarized the current understanding of the role of various miRNAs in TGF-β and BMP-regulated signaling pathways (Tables 15.1 and 15.2). MiRNAs control nearly all aspects of cell biology, including cell death, migration, proliferation, and differentiation. MiRNAs upstream or downstream of TGF-β and BMP signaling pathways are keys to the differentiation and maintenance of the contractile phenotype of VSCM exhibited by a decrease in proliferation and an increase in contractile markers. Aberrant activities of TGF-β or BMP are associated with an array of cardiovascular diseases, and there is a critical need to modulate the activities of TGF-β and BMP signaling to prevent or ameliorate cardiovascular diseases. Since TGF-β and BMP have a variety of essential functions in different tissues, targeting a core molecule in the TGF-β or BMP signaling pathway is not ideal for the therapeutic approach. We speculate that the modulation of the levels of TGF-β or BMP-regulated miRNAs or miRNAs that target Smads could be a better therapeutic option because many miRNAs are expressed in a tissue-specific manner and, therefore, may exhibit minimal side effects in other tissues or organs. Preliminary studies in animal models illustrate that tail vein or subcutaneous injection of chemically modified stable short RNA oligonucleotides with sense or antisense miRNA sequence can be delivered to various organs and modulate gene expression in the recipient tissues (Montgomery et al. 2011). In one study, subcutaneous injection of an antagomir to miR-208a, whose expression is known to be induced upon heart failure, improved cardiac function, overall health, and survival after ingesting a high salt diet previously shown to cause hypertension (Montgomery et al. 2011; Rodenbaugh et al. 2007). Thus, miRNAs might be a viable therapeutic option for cardiovascular diseases. Further studies on miRNAs related to the TGF-β signaling pathways will grant a better understanding of the role of these pathways in cardiac development and homeostasis and, hence, offer potential for miRNA-based therapies.

Table 15.1 TGF-β/BMP regulated miRNAs

miRNA	Regulation	Biological activities of miRNA
miR-143/145	TGF-β ↑ BMP ↑	TGF-β/BMP induces myocardin/ MRTFs which leads to transcriptional activation of miR-143/145 (Cordes et al. 2009; Xin et al. 2009). MiR-143/145 downregulates KLF4, KLF5, and ELK1, promoting the contractile phenotype of VSMC (Davis-Dusenbery et al. 2011; Rangrez et al. 2011)
miR-21	TGF-β ↑ BMP ↑	TGF-β/ BMP stimulation increases processing of pri-miR-21 to pre-miR-21 through association with p68 and Drosha (Davis et al. 2008) MiR-21 targets PDCD4 and DOCK4/5/7 to promote the contractile phenotype of VSMC (Davis et al. 2008; Kang et al. 2012)
miR-29	TGF-β ↓	TGF-β transcriptionally downregulates miR-29 expression through Smad3 (Xiao et al. 2012). MiR-29 targets many collagen genes and, therefore, decreases expression of miR-29 by TGF-β increases expression of ECM genes (Xiao et al. 2012)
miR-125b	TGF-β ↑	TGF-β transcriptionally induces miR-125b expression (Ghosh et al. 2012). MiR-125b targets p53. p53 blocks type I collagen synthesis and Smad3-dependent collagen gene transcription (Ghosh and Vaughan 2012)
miR-27b	TGF-β ↓	TGF-β transcriptional downregulated miR-27b expression (Wang et al. 2012c). MiR-27b targets PPARγ, which protects against cardiac hypertrophy (Wang et al. 2012c)
miR-155	TGF-β ↑	MiR-155 is transcriptionally induced by TGF-β (Kong et al. 2008)
miR-24	TGF-β ↑	TGF-β transcriptionally induces miR-24 expression (Dogar et al. 2011). However, miR-24 target Furing and reduces maturation of TGF-β ligand, creating a feedback mechanism (Wang et al. 2012b)

Table 15.2 MiRNAs regulating TFG-β/ BMP signaling

miRNA	Regulation	Activity by TGF-β/BMP
miR-155	↓ TGF-β	MiR-155 targets Smad2 and AT1R (Kong et al. 2008) MiR-155 is also transcriptionally induced my TGF-β (Kong et al. 2008)
miR-26a	↓ TGF-β ↓ BMP	MiR-26a targets Smad1 and Smad4 (Leeper et al. 2011). MiR-26a also targets PTEN, thereby promoting the synthetic phenotype of VSMC (Huse et al. 2009)
miR-200	↓ TGF-β	MiR-200a/b/c prevents TGF-β-induced EMT (Yang et al. 2012). The miR-200 family of miRNAs targets GATA3, ZEB1, and ZEB2 to prevent increased transcription of mesenchymal markers (Gemmill et al. 2011). ZEB1 and ZEB2 suppress expression of miR-200.
miR-24	↓ TGF-β ↓ BMP	MiR-24 targets Furin and reduces maturation of TGF-β ligand (Wang et al. 2012b). However, TGF-β induces miR-24 expression, creating a feedback mechanism (Dogar et al. 2011) MiR-24 targets Trb3, preventing signaling through the BMPRII and promoting the synthetic phenotype in VSMC (Chan et al. 2010)

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Part IV
Other Diseases

Chapter 16
TGF-β and Genetic Skeletal Diseases

Shiro Ikegawa, Mitsuko Nakashima, and Naomichi Matsumoto

Abstract Transforming growth factor-β (TGF-β) is a multi-functional growth factor that controls proliferation, differentiation, and other cellular functions in many types of cells. TGF-β and its family molecules play critical roles in development and maintenance of the skeleton, which are underlined by various skeletal phenotypes caused by mutations in genes encoding ligands, receptors, and signaling molecules of the TGF-β family (TGF-β-pathy). Genetic diseases are categorized as monogenic and polygenic diseases. They give us clues to understand mechanisms for development and maintenance of tissues and organs in humans, which is also true to skeleton. In this paper, we review representative monogenic and polygenic TGF-β-pathy of the skeleton with focus on their significance for understanding mechanisms regulating the skeletal system.

Keywords Asporin • Bone • Cartilage • Cartilage intermediate layer protein (CILP) • Extracellular matrix • Growth factor • Monogenic disease • Polygenic disease • Susceptibility gene • TGF-β-pathy

Abbreviations

AKT	v-akt murine thymoma viral oncogene homolog
BMD	Bone mineral density
BMSC	Bone marrow stromal cell

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CED	Camurati–Engelmann disease
CILP	Cartilage intermediate layer protein
D-repeat	Aspartic acid repeat
ECM	Extracellular matrix
LAP	Latency-associated peptide
LDD	Lumbar disc disease
LDS	Loeys–Dietz syndrome
LRR	Leucine-rich repeat
MAPK	Mitogen activated kinase-like protein
OA	Osteoarthritis
OPLL	Ossification of the posterior longitudinal ligament of the spine
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
ROCK	Rho-associated protein kinase
SLRP	Small leucine-rich proteoglycan

16.1 Role of Transforming Growth Factor-β in Skeletal Tissue

Transforming growth factor-β (TGF-β) is a multifunctional growth factor that controls proliferation, differentiation, and other cellular functions in many types of cells, playing critical roles in development and maintenance of many types of tissues and organs. It is true in the skeleton (skeletal tissue). TGF-β stimulates proliferation and differentiation of osteoblast (bone forming cell) and chondrocyte (cartilage forming cell) in vitro. High levels of *TGFB1* (gene for TGF β1) mRNA and its protein are found in developing cartilage (growth cartilage) and endochondral and membrane bones, suggesting its role in growth, differentiation, and maintenance of the skeleton (Dickinson et al. 1990). The critical roles of *TGFB* and its family genes to the human skeletal system are underlined by various skeletal phenotypes of patients with diseases caused by mutations in genes encoding ligands, receptors, and signaling molecules of the TGF-β family (Table 16.1). These TGF-β related diseases (TGF-β-pathy) form an important subgroup of skeletal dysplasia (Warman et al. 2011).

16.2 TGF-β-Pathy

Genetic diseases are categorized as monogenic and polygenic diseases from their involvement of the gene. The monogenic (Mendelian) disease is caused by a mutation of a single gene (disease gene). The effect of the disease gene to the disease phenotype is decisive in monogenic diseases. Its inheritance is relatively simple and predictable, following Mendel's law. In contrast, the polygenetic disease is multifactorial; its phenotype is determined by combined and concerted effects of a group of genes (susceptibility genes) and the environment. Its inheritance is complex.

Table 16.1 Monogenic diseases of the skeleton caused by mutations of TGF-β and TGF-β related genes

Disease[a]	Inheritance	OMIM ID	Disease gene
Diaphyseal dysplasia Camurati–Engelmann	AD	131300	*TGFB1*
Marfan syndrome (familial thoracic aortic aneurysms and dissection)	AD		*TGFB2*
Brachydactyly type A2	AD	112600	*BMP2*
Grebe dysplasia	AR	200700	*GDF5*
Fibular hypoplasia and complex brachydactyly (Du Pan)	AR	228900	*GDF5*
Brachydactyly type A2	AD	112600	*GDF5*
Brachydactyly type C	AD, AR	113100	*GDF5*
Multiple synostoses syndrome type 2	AD	186500	*GDF5*
Proximal symphalangism type 2	AD	185800	*GDF5*
Klippel–Feil anomaly with laryngeal malformation	AD	148900	*GDF6*
Loeys–Dietz syndrome types 1A, 2A	AD	609192, 610168	*TGFBR1*
Loeys–Dietz syndrome types 1B, 2B	AD	608967, 610380	*TGFBR2*
Acromesomelic dysplasia with genital anomalies	AR	609441	*BMPR1B*
Brachydactyly type A2	AD	112600	*BMPR1B*
Fibrodysplasia ossificans progressiva	AD	135100	*ACVR1*
Loeys–Dietz syndrome type C (aortic aneurysms and dissections with early-onset OA)	AD	613795	*SMAD3*
Myhre syndrome	AD	139210	*SMAD4*
Geleophysic dysplasia	AR	231050	*ADAMTSL2*
Osteopoikilosis/Buschke–Ollendorff syndrome	AD	166700	*LEMD3*
Melorheostosis with osteopoikilosis	AD	155950	*LEMD3*
Sclerosteosis	AR	269500	*SOST*
Endosteal hyperostosis, van Buchem type	AR	239100	*SOST*
Diaphanospondylodysostosis	AR	608022	*BMPER*
Multiple synostoses syndrome type 1	AD	186500	*NOG*
Proximal symphalangism type 1	AD	185800	*NOG*
Cenani–Lenz like syndactyly	Sporadic	212780	*GREM1, FMN1*

[a]Disease names are based on "Nosology and classification of genetic skeletal disorders: 2010 revision" (Warman et al. Am J Med Genet 2011)

OA osteoarthritis, *AD* autosomal dominant, *AR* autosomal recessive, *ADAMTSL2* ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs)-like 2, *LEMD3* LEM domain-containing 3, *SOST* sclerostin, *BMPER* bone morphogenetic protein-binding endothelial cell precursor-derived regulator, *NOG* noggin, *GREM1* gremlin 1, *FMN1* formin 1

TGF-β-pathy covers both categories of genetic diseases involving the skeletal system. These diseases give us a clue to clarify the roles of TGF-β in skeleton in vivo in human as well as physiological mechanisms controlling the skeletal system.

As their numbers are so many, this review focuses on a limited number of TGF-β-pathy affecting the skeleton. The list of monogenic diseases includes Camurati–Engelmann disease (CED; MIM #131300) and Loeys–Dietz syndrome (LDS; MIM #609192). Polygenic diseases related to TGFB are osteoporosis (MIM #166710),

otosclerosis, ossification of the posterior longitudinal ligament of the spine (OPLL; MIM #602475), osteoarthritis (OA, MIM 165720), and lumbar disc disease (LDD). They all are common diseases.

16.3 Monogenic Diseases

16.3.1 Camurati–Engelmann Disease

16.3.1.1 Clinical Features

CED (alias: Engelmann disease, progressive diaphyseal dysplasia) is a rare skeletal dysplasia of autosomal dominant inheritance. CED is characterized by the cortical thickening of the diaphyses of the long bones. Endo- and exo-osteal bone formations are increased (Fig. 16.1a). Increased exo-osteal bone formation (hyperostosis) is bilateral and symmetrical and usually starts at diaphyses of femora or tibiae, expanding to other long tubular bones (Fig. 16.1b, c). As the disease progresses, metaphyses of the bones may be affected as well, but their epiphyses are spared. Sclerotic changes at the skull base are frequently present. Interestingly, the onset of the disease is usually during childhood and almost always before age 30, indicating that the disease process has nothing to do with prenatal and early postnatal skeletal development. Most patients present with limb pain and muscular weakness of the affected limbs, a waddling gait and easy fatigability. The patients present with apparent undernutrition (Marfanoid habitus) (Fig. 16.1d). The muscular weakness is not necessarily progressive and typical bone changes may be found in asymptomatic individuals. Systemic manifestations including anemia, leukopenia, and hepatosplenomegaly occur occasionally (Janssens et al. 2006).

16.3.1.2 Molecular Genetics of CED

CED results from domain-specific heterozygous mutations in *TGFB1*. The group led by Norio Niikawa mapped the CED locus to 19q13.1–q13.3 by a parametric linkage analysis (Ghadami et al. 2000) and subsequently detected heterozygous missense mutations in exon 4 of *TGFB1*, near the carboxy terminus of the latency-associated peptide (LAP) (Kinoshita et al. 2000). Janssens et al. (2000) confirmed the discovery by finding the same and similar mutations in the LAP region as well as other types of mutations. Different mutations affecting the LAP region have been reported (Kinoshita et al. 2004). Genetic heterogeneity is known in CED; i.e., patients with similar bone phenotypes but with no *TGFB1* mutation are considered as CED type II (MIM 606631).

These LAP mutations cause increased TGF-β function in CED. The mutations are predicted to cause LAP insufficiency due to conformational changes of the LAP proteins by affecting their cysteine residues necessary for di-sulfide bond formation. In vitro studies show these mutations result in disrupted association of LAP and

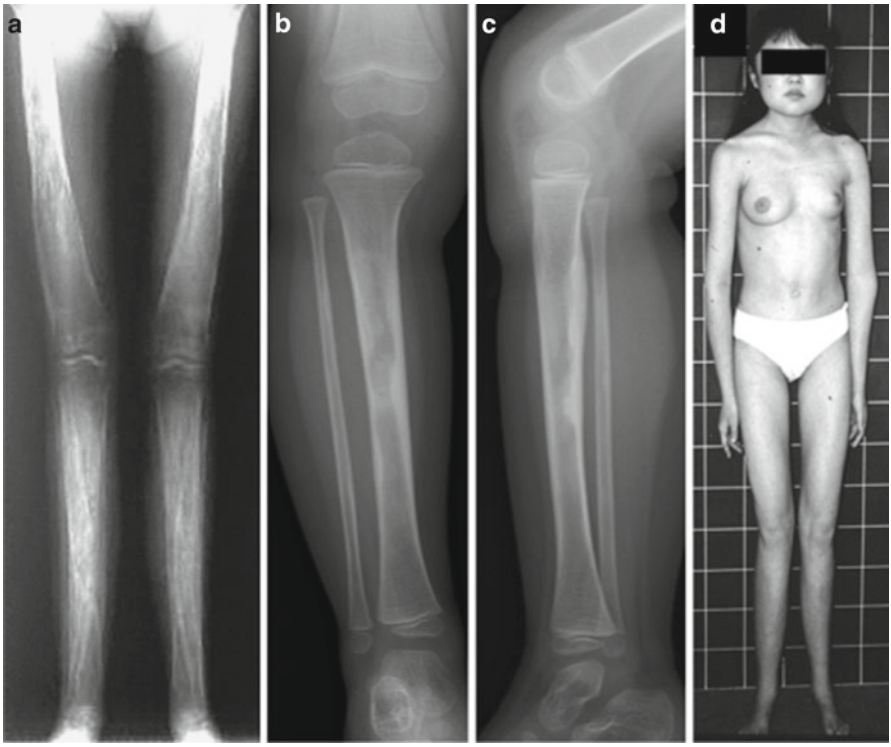

Fig. 16.1 Clinical features of Engelmann disease. (**a**) radiographs of the lower extremities in the late stage bone changes of the Engelmann disease. Symmetric hyperossification of the long bones. Bone widths and bone densities are increased, showing that both exo- and endo-osteal bone formation are increased. (**b**, **c**) the *right* leg A-P (**b**) and lateral (**c**) radiographs showing the early stage bone changes of disease. Irregular thickening of the cortex of tibial diaphysis. (**d**) marfanoid habitus (slender and relatively long limbs) of a patient

TGF-β1 and the subsequent release of the mature (active) TGF-β1 followed by increased TGF-β signaling (Saito et al. 2001). The cell growth of fibroblasts from a CED patient and growth of the mutant gene-transfected fibroblasts was suppressed by blocking TGF-β signaling. Janssens et al. (2003) investigated the effects of the CED mutation using a luciferase reporter assay specific for TGFB-induced transcriptional response. They showed that the mutations increase TGF-β1 activity by increasing active TGF-β1 in the medium of the transfected cells. Thus, the pathomechanism of CED is hyperactivation of TGF-β1 due to LAP insufficiency.

16.3.1.3 Significance of CED—Identification of the "Coupling Factor"

Skeletal phenotypes and mutations of CED gave a hint to solve a long-sought mystery of bone metabolism, "coupling"—a synchronized transition from bone resorption to bone formation in adult bone. Bone is a highly dynamic and specialized

organ, constantly adjusting its structure in response to mechanical and metabolic stimulation such as exercise, gravity, and calcium depletion. This process is called "remodeling," where old useless bone is first removed by osteoclasts, bone-resorbing cells of hematopoetic origin, and then new necessary bone is rebuilt by osteoblasts, bone-forming cells of mesenchymal origin. To maintain the structural and functional integrity of bone through this process, the steps of destruction and formation must be coordinated spatiotemporally and quantitatively. The coordination that synchronized the opposing activities is called "coupling" and its failure results in pathological states, including osteoporosis (decreased bone) and osteopetrosis (increased, but functionally useless bone), both of which produce mechanically weak bone liable to fracture. Therefore, its molecular basis has been one of the main research topics in skeletal biology and research of bone and joint diseases; however, even the key molecule(s) controlling it remains enigmatic.

16.3.1.4 TGF-β is the "Coupling" Factor

The TGF-β1 effect in bone metabolism in vivo has long been paradoxical (Iqbal et al. 2009). TGF-β1 stimulates osteoblast proliferation and differentiation in vitro; however, over-expression of TGF-β1 stimulates bone turnover, resulting in bone loss (high-turnover osteoporosis) (Erlebacher et al. 1998). Conversely, blocking of TGF-β signaling causes decreased bone degradation, resulting in excessive bone formation with normal bone formation rates (Filvaroff et al. 1999).

Tang et al. (2009) generated double knockout (KO) mice of *Tgfb1* and the recombination-activating gene-2 (Rag2) because *Tgfb1*–KO mice (single KO mice) develop autoimmune disease and are unavailable for postnatal bone metabolism studies. They showed that TGF-β1 is necessary for the localization of bone marrow stromal cells (BMSCs) and osteoblast precursor cells to sites of bone resorption. TGF-β1 is stored in bone as an inactivated form by binding to LAP. Osteoclasts release and activate TGF-β1 by secreting bone catabolizing enzymes and acid pH during bone resorption. Activated TGF-β1 guides BMSCs correctly to sites of resorption through a Smad signaling pathway. Thus, the coupling signal is valid only at sites of bone resorption. The switch turns on at the right place at the right time. In CED, the mutations deliver active TGF-β1 regardless of the demand of mechanical stress and excessive bones are formed for no purpose. Thus, CED provides an in vivo model to validate the role of TGF-β1 as the "coupling" factor that coordinates bone resorption and formation.

16.3.2 Loeys–Dietz Syndrome

16.3.2.1 Clinical Features

LDS, previously known as Marfan syndrome type 2, is an autosomal dominant syndrome characterized by aortic aneurysm with multiple systemic involvements. Two types are known; LDS type 1 shows a triad of arterial tortuosity and aneurysm,

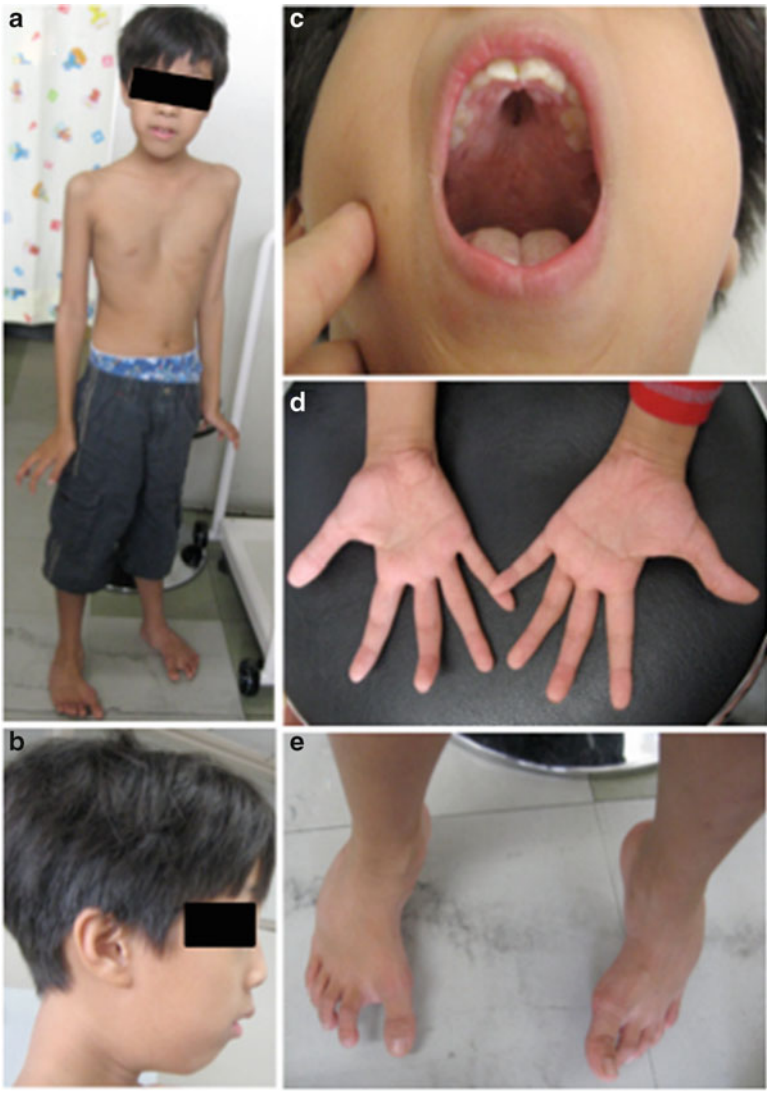

Fig. 16.2 Clinical pictures of Loeys–Dietz syndrome. A patient harboring a *TGFBR2* (for type 2 TGF-β receptor) mutation. (**a**) Slender constitution, pectus excavatum, and dolichostenomelia. (**b**) Dolichocephaly with retrognathia. (**c**) cleft palate (after surgery). (**d**) long and slender fingers. (**e**) talipes equinovarus and sandal gap

hypertelorism, and bifid uvula or cleft palate (Fig. 16.2), while LDS type 2 has only bifid uvula. LDS may lead to intellectual disability. LDS type 1 exhibits intracranial abnormalities, including Chiari malformation type I and hydrocephalus. The natural history of LDS is defined by aggressive arterial aneurysm and dissection.

LDS show various skeletal features (Fig. 16.2). Patients are commonly slender with characteristic dolichostenomelia and arachnodactyly. They have various limb abnormalities including joint laxity, foot deformities (talipes planus, valgus, varus, and equinovarus), hammer toes, and post-axial polydactyly. They also present with spinal and rib abnormalities, cervicospinal instability, scoliosis, and pectus carinatum or excavatum. Other skeletal features include craniosynostosis, retrognathia, malar hypoplasia, protrusio acetabulae, skeletal fragility, and osteoporosis (Kalra et al. 2011; Loeys et al. 2005, 2006).

16.3.2.2 Molecular Genetics

Collod et al. (1994) performed a linkage analysis in a large LDS family without the fibrillin 1 gene (*FBN1*) mutations and found a candidate locus LDS at 3p24.2-p25. Mizuguchi et al. (2004) identified the disruption by chromosomal abnormality of *TGFBR2* encoding the type 2 TGF-β receptor in a Japanese individual and found heterozygous *TGFBR2* mutations in French and Japanese LDS families. Loeys et al. (2005) found *TGFBR2* and *TGFBR1* mutations in LDS. Germline mutations of *TGFBR1* and *TGFBR2* are also found in patients with familial thoracic aortic aneurysms and their dissection demonstrated lack of any skeletal deformities (Matyas et al. 2006; Pannu et al. 2005). As *TGFBR1* and *TGFBR2* are also known as tumor suppressor genes, correlation of these hereditary disorders with carcinogenesis is suspected; however, multiple self-healing squamous epithelioma, which is caused by disease-specific *TGFBR1* mutations, is the only evidence of correlation between the *TGFBR* germline mutation and tumor predisposition so far (Goudie et al. 2011).

16.3.2.3 Significance—Non-canonical TGF-β Signaling Pathway

TGF-β signaling is mediated by Smad-dependent (canonical) and Smad-independent (non-canonical) pathways. The latter includes PI3K-AKT, ROCK, and MAPK (p38, JNK and RAS-ERK) (Ikushima and Miyazono 2010). Horbelt et al. (2010) investigated *TGFBR2* mutations in LDS and found that they show a dominant-negative effect in which the receptor protein expression and Smad2 phosphorylation were reduced in vitro. Although the pathomechanisms of skeletal abnormalities in LDS are far from being understood, studies of aortic aneurysms shed light on possible roles of TGF-β signaling in LDS. Most LDS mutations are missense changes at the serine-threonine kinase domain of *TGFBR1* and *TGFBR2*, which were supposed to result in impaired TGF-β signaling. However, histopathological studies of aortic tissues from LDS patients revealed paradoxical increase in TGF-β signaling with enhanced expression of connective tissue growth factor and collagen deposition. Intra-nuclear phosphorylated Smad2 expression was also increased (Holm et al. 2011; Loeys et al. 2005, 2006). Systemic administration of a TGF-β neutralizing antibody in *Fbn1*-deficient MFS mice showing similar manifestations to LDS can prevent the aberrant enlargement of the aortic vessels (Habashi et al. 2006; Ng et al. 2004). *ERK1/2* were also activated and Losartan, the angiotensin II type 1 receptor

(AT1) blocker, can prevent aortic aneurysms by inhibiting *ERK* pathway in MFS mice (Habashi et al. 2006, 2011). Furthermore, expression of *TGFB2* and *TGFBR3* proteins was elevated and the Smad-independent p38 MAPK pathway was activated in *Tgfbr2* mutant mice (Iwata et al. 2012). These observations implied that not only the canonical signaling but also the non-canonical signaling has an important role in the pathogenesis of LDS.

16.4 Polygenic Diseases

Case–control association studies indicate that *TGFB* is involved in several polygenic diseases affecting the skeleton.

16.4.1 Osteoporosis

Osteoporosis is the pathological decrease of the bone tissue that leads to an increased risk of fracture, mainly in spine (vertebral body), distal radius, and femoral neck. It is represented as reduction of bone mineral density (BMD), but the basic abnormality is a decrease in bone matrix proteins, not in mineralization. Osteoporosis has several clinical types and is classified as primary and secondary osteoporosis based on its etiology. Primary osteoporosis consists of postmenopausal and senile osteoporosis. Postmenopausal osteoporosis is the most common bone and joint disease in women after menopause. Secondary osteoporosis results from various diseases relating to bone metabolism, or prolonged use of medications such as glucocorticoids.

Genetic association of postmenopausal osteoporosis and *TGFB1* has been known. A group reported a significant association of a missense variant in *TGFB1*, c.29T>C (p.Leu10Pro) with BMD and frequency of vertebral fractures in postmenopausal women (Yamada et al. 1998). (Note. c.29T>C: The change of C (cytosine) nucleotide at the position 788 in the cDNA sequence of the gene to T (thymine). p.Leu10Pro: The missense change of Thr (threonine) at position 263 of the protein to Ile (isoleucine)). The group also reported a correlation of the serum concentration of TGF-β1 with the c.29T>C genotype. However, statistical significance of the association is not definite because of the small number of subjects used for the study and of multiple hypothesis testing. Subsequent large-scale association studies in Japanese (Kou et al. 2011) and other ethnic populations have not replicated the association.

16.4.2 Otosclerosis

Otosclerosis is a common bone dysplasia that is unique to the endochondral layer of the otic (labyrinthine) capsule. It is a major cause of acquired hearing loss in adult

life, leading to a progressive hearing loss due to stapes fixation. The mean age of its onset is in the third decade (under 50 years of age for 90 % of patients). Genetic heterogeneity of otosclerosis is present; both monogenic and polygenic forms of this disorder are known.

Association of a missense variation in *TGFB1*, c.788C>T (p.Thr 263Ile) with otosclerosis susceptibility has been reported in western European populations (Thys et al. 2007). The association could be replicated in two independent populations (Thys & Camp 2009). A reporter assay showed that the susceptibility variant (Thr 263) has lower Smad signaling activity, although the functional impact of this variant to the pathogenesis of otosclerosis remains unclear. Notably, association of *BMP2* and *BMP4* with otosclerosis is also reported (Thys & Camp 2009).

16.4.3 Ossification of the Posterior Longitudinal Ligament of the Spine

The posterior longitudinal ligament of the spine is a ligament situated at the posterior side of the spinal cord in the spinal canal, a space formed by vertebral bones. OPLL is a disease caused by abnormal (ectopic) ossification of the ligament. OPLL is a localized hyperostosis of this specific ligament, but systemic tendency of abnormal ossification has been reported in a certain proportion of the patients. The incidence of OPLL is 1.9–4.3 % in Japan (Stapleton et al. 2011; Matsunaga and Sakou 2012), and comparable incidence has been reported in other countries, especially in East Asia (Saetia et al. 2011). OPLL causes serious neurological disturbances due to compression of the spinal cord and the nerve roots by the ossified ligament. Its average age of onset is over 50 years with male predominance, affecting motility and quality of life in the patients.

By a case–control study for a group of candidate genes in Japanese OPLL, Horikoshi et al. (2006) found significant association between an intronic SNP in the *TGFB3* gene (rs2268624) and OPLL susceptibility. The statistical significance of the association is not definite to the present standard of the association study, and the functionality of the sequence variation remains unclear. The association data should be checked for their replication in different populations, preferably of different ethnic populations.

16.4.4 Regulatory Molecules of TGF-β in the Pathogenesis of Common Bone and Joint Diseases

16.4.4.1 Extracellular Matrix

Skeletal tissues have large amounts of extracellular matrix (ECM). The primary function of ECM in the skeleton is its contribution to physical and biomechanical

strength; however, the ECM also plays important biological roles in skeletal metabolism through regulation of growth factors, including TGF-β (Grimaud et al. 2002; Chen et al. 2004). Many ECM proteins bind to TGF-β and modulate its function. The ECM proteins are mostly tissue-specific, playing a critical role in the tissue-specific, context-dependent function of TGF-β. Not a few of them are associated with common bone and joint diseases.

16.4.4.2 Asporin and Osteoarthritis

Osteoarthritis

Joint is an organ for locomotion and is composed by various tissues including cartilage, bone, synovium, and ligament. OA is the most common disease of the joint, affecting more than 10 % of the population in developed countries all over the world. OA can affect any synovial joint in the human body, mainly in weight-bearing joints of the lower extremities. Histopathologically, OA is characterized by degeneration of articular cartilage that covers the surface of the joint. OA can be resulted from definite causes such as trauma and hereditary and metabolic diseases (secondary OA), but most of them are idiopathic (primary OA), in which genetic factors have been implicated (Dai and Ikegawa 2010). Patients with OA suffer from pain and limitations to the movement of their affected joints. OA is a primary cause of decreased activity in daily living and quality of life after middle age.

Asporin

Asporin is an ECM protein first identified as a specific protein extracted from articular cartilage (Lorenzo et al. 2001). Asporin is a member of the small leucine-rich proteoglycan (SLRP) family (Hocking et al. 1998), which belongs to the leucine-rich repeat (LRR) super-family of proteins. The SLRP family is classified into three classes based on similarities in protein sequence, conserved spacing of the four cysteine cluster in their N-terminal domain, and genomic structure (Iozzo & Murdo 1996; Ameye and Young 2002). Asporin belongs to class I SLRPs which contains ten repeats of the LRR motif, L-X-X-L-X-L/I-X-X-N-X-L/I, in their core proteins (Scholzen et al. 1994; Lorenzo et al. 2001). The N-terminal regions of other class I SLRPs, including decorin and biglycan carry a few chondroitin or dermatan sulfate chains, respectively (Krusius and Ruoslahti 1986; Fisher et al. 1989), whereas asporin contains no consensus sequences for glycosaminoglycan attachment. Therefore, asporin is not a proteoglycan unlike other class I SLRPs. Asporin has a unique aspartic acid repeat (D-repeat) in the N-terminal instead (Henry et al. 2001; Lorenzo et al. 2001).

Association of Asporin with OA

SLRP family genes have been implicated in the etiology of OA. KO mice of fibromodulin (Gill et al. 2002) and double KO mice of fibromodulin and biglycan

(Ameye et al. 2002) develop mild and severe premature OA, respectively. Asporin mRNA is expressed abundantly in OA articular cartilage (Lorenzo et al. 2001; Kizawa et al. 2005). These observations prompted our group to examine the asporin gene (*ASPN*) as a candidate gene for OA.

We made a list of common polymorphisms in *ASP* and carried out a large-scale case–control association study in Japanese OA populations. We identified a significant association between *ASPN* and OA (Kizawa et al. 2005). The D-repeat of asporin is encoded by a polymorphic triplet (GAT) repeat. In the Japanese variants of asporin, there are 10–19 D-repeats. Among them, the allele that encodes 14 repeats (D14 allele) was significantly overrepresented in OA patients. The association was replicated in Han Chinese (Jiang et al. 2006) and European Caucasian populations (Nakamura et al. 2007).

Asporin and TGF-β

SLRPs such as decorin and biglycan have been shown to bind TGF-β and control its accessibility to the receptors (Border et al. 1992; Isaka et al. 1996). A series of experiments using the ATDC5 cell, an in vitro model of chondrocyte differentiation in mouse (Shukunami et al. 1996) and human cartilage cells revealed that asporin co-localizes with TGF-β1 on the cell surface, binds to TGF-β1, and blocks the binding of TGF-β1 to the type II TGF-β receptor outside of the cell. Consequently, phosphorylation of Smads, mainly Smad3 is inhibited and expression of the cartilage marker genes downstream of the TGF-β-Smad signal, including those for type II collagen and aggrecan are decreased (Kizawa et al. 2005; Kou et al. 2007). Knockdown of asporin by siRNA in chondrocyte increases the cartilage marker genes (Nakajima et al. 2007). Thus, asporin works as a negative regulator of chondrocyte differentiation and cartilage ECM formation through negative regulation of *TGFB1*. On the other hand, the group also showed that TGF-β1 could stimulate asporin gene expression, implicating asporin and TGF-β1 into a functional feedback loop that controls homeostasis of the articular cartilage.

Mechanism Causing Susceptibility and Role of Asporin in Articular Cartilage Metabolism

In vitro transfection experiments examining inhibitory effects of different alleles of asporin showed that the D14 asporin variant had the strongest inhibitory effect on TGF-β signaling. Individuals who have the susceptibility allele are suspected to have decreased potential of cartilage recovery and are hence susceptible to OA.

Joints are subjected to daily noxious stimuli. Wear and tear by daily weight bearing and minor or major injuries during daily activity usually cause joint damage. To recover from the damage, cell proliferation and matrix production are necessary. Many growth factors and cytokines support the process. In cartilage, TGF-β is the cardinal player leading to regeneration. Many molecules would enhance TGF-β and its function. However, too much function of TGF-β would result in

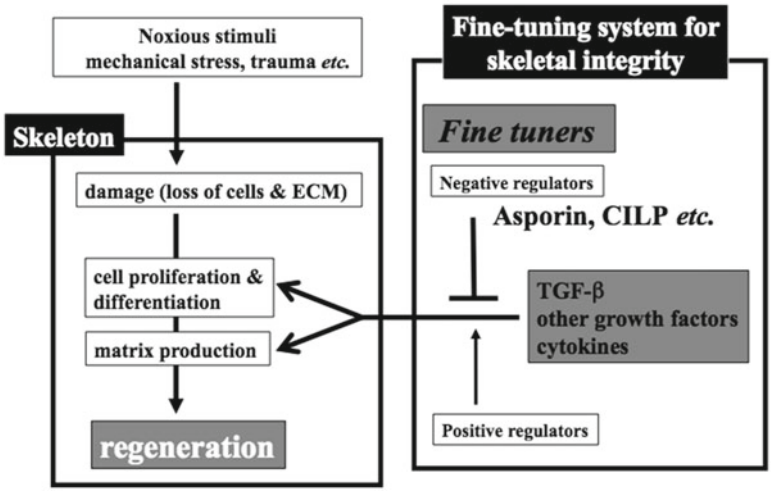

Fig. 16.3 A fine-tuning system for maintaining skeleton. The fine-tuners listed here (asporin and CILP) primarily function through sequestration of TGF-β ligands. They operate at distinct locations, leading to distinct diseases

unwanted results such as non-smooth joint surface and cartilage tumor formation. Therefore, restriction of TGF-β function in appropriate level is necessary. We suppose that asporin is central to the control and fine-tuning of TGF-β function in articular cartilage (Fig. 16.3). The aberration of the fine-tuning system would lead to degeneration.

16.4.4.3 Cartilage Intermediate Layer Protein and LDD

Lumbar Disc Disease

LDD results from degeneration of intervertebral discs of the lumbar spine. LDD is one of the most common musculoskeletal disorders. LDD is a significant health care issue all over the world. Disc degeneration and concomitant disc herniation are a major cause of low back pain and leg pain. Low back pain affects 70–85 % of all people during their lifetime, and lumbar disc herniation is the most common cause of activity limitation in individuals younger than 45 years of age (Andersson 1999). The etiology and pathogenesis of LDD is largely unknown; however, significant roles for genetic factors in the etiology of LDD are indicated by epidemiological studies (Battie et al. 1995; Sambrook et al. 1999).

Cartilage Intermediate Layer Protein (CILP)

CILP is an ECM protein first identified in the intermediate (middle) zone of human articular cartilage (Lorenzo et al. 1998). CILP also localizes to meniscus, tendon,

ligament, synovial membrane, and intervertebral disc. Its expression increases substantially in association with aging in human articular cartilage. CILP is one of the few cartilage ECM proteins whose expression is up-regulated in early and late stages of OA (Lorenzo et al. 1998, 2004). *CILP* expression levels were high in human intervertebral discs and increased as disc degeneration progressed (Seki et al. 2005).

Association of CILP with LDD

Several lines of evidence show that ECM proteins play crucial roles in intervertebral disc homeostasis. The association with LDD has been reported for the genes encoding collagen IX (*COL9A2* (Annunen et al. 1999) and *COL9A3* (Paassilta et al. 2001)) and aggrecan (Kawaguchi et al. 1999). These observations underscore the importance of ECM protein metabolism in the etiology of LDD. Therefore, we examined candidate genes encoding ECM proteins of intervertebral discs and found a significant association between a missense SNP, c.1184T>C (p.Ile395Thr) in the *CILP* gene and LDD.

TGF-β in Intervertebral Disc Metabolism and Etiology of LDD

The TGF-β signal is critical for metabolism of the intervertebral disc in vivo. Cells immunopositive for TGF-β1 and the TGF-β type II receptor are present in increased numbers in herniated discs (Tolonen et al. 2001; Specchia et al. 2002). Transgenic mice harboring a dominant-negative TGF-β type II receptor develop skeletal abnormalities and OA (Serra et al. 1997), while *Smad3* KO mice develop joint degeneration and spinal deformity, caused by loss of articular cartilage and decreased production of proteoglycans (Yang et al. 2001). Further, TGF-β1 treatment decreases the expression of matrix metalloproteinases that are involved in tissue remodeling events in disc degeneration (Pattison et al. 2001). Interestingly, association of asporin with LDD is also reported (Song et al. 2008).

Similar to asporin in articular cartilage and OA, CILP functions as a modulator for TGF-β activity in intervertebral disc tissue. CILP bound to TGF-β1 and inhibited TGF-β1-mediated induction of ECM genes in the intervertebral disc (Seki et al. 2005). p.Thr395 CILP produced from the disease-susceptible allele of CILP suppressed TGF-β1-induced gene expression more efficiently than p.Ile395 CILP produced from the non-susceptible allele. The aberrantly increased inhibitory effects of CILP attributed to the susceptibility allele would perturb the balance of TGF-β control over chondrocyte metabolism and intervertebral disc tissue maintenance, leading to a susceptibility to LDD caused by inadequate response of intervertebral disc cells to injury and mechanical stress. Just as in articular cartilage, the fine-tuning system (Fig. 16.3) is necessary in vertebral discs.

16.4.4.4 General Concept: TGF-β-ECM System

ECM proteins expressed abundantly and specifically in skeletal tissues and cells. They bind to TGF-β and positively or negatively regulate TGF-β activity, controlling skeletal tissue metabolism. Then, their abnormalities could cause susceptibility to common bone and joint diseases. The association study underscores the importance of TGF-β in development and maintenance of human skeletal tissue and further highlights the functional link between TGF-β, ECM proteins, and the bone and joint diseases. Therefore, agents controlling and modifying the TGF-β-ECM system would be promising targets for treatment, presenting a novel therapeutic strategy for common bone and joint diseases.

16.5 Further Questions

Among the three isoforms of TGF-β in human, our micro-array data show *TGFB1* is a predominant form in articular cartilage (Ikegawa et al. unpublished data). High levels of *TGFB1* mRNA and protein are found in developing cartilage, endochondral and membrane bones, suggesting its role in the growth and differentiation of bone and cartilage (Dickinson et al. 1990). Although the amino-acid sequence homologies of *TGFB2* and *TGFB3* to *TGFB1* are only 74 % and 77 %, respectively, they have been considered to work in similar ways to *TGFB1*. Their in vivo functions in the human skeleton are unclear.

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Chapter 17
TGF-β in Brain Disorders

Kerstin Krieglstein

Abstract Transforming growth factor beta (TGF-β) is known to regulate numerous cell functions in the nervous system development, adult maintenance, and degeneration. TGF-β carries roles in neurons and glia and is involved in the regulation of proliferation, differentiation, neuron survival and death, as well as orchestrating its response to lesion. In the context of brain disorders the current understanding of TGF-β action is discussed for brain tumors, neurodegenerative disease, such as Alzheimers' and Parkinson's disease, in insults such as ischemia, stroke, and vascular damage, as well as changes in neuronal activity, such as hyperactivity as seen in epilepsy, or in neuronal depression.

Keywords Cell cycle regulation • ECM • Neuroprotection • Cell death • Blood • Neurons • Astrocytes • Microglia • Myelination • Neurogenesis

Abbreviations

Aβ	Amyloid β
AD	Alzheimer's disease
Alk	Activin-receptor like kinase
ALS	Amyotrophic lateral sclerosis
APP	Amyloid precursos protein
BBB	Blood–brain barrier
BDNF	Brain-derived neurotrophic factor
Cdk	Cyclin-dependent kinase
CNS	Central nervous system

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CNTF	Ciliary neurotrophic factor
CSF	Cerebrospinal fluid
E	Embryonic day
ECM	Extracellular matrix
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
Fox	Forkhead box
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GFRα	GDNF receptor
Id4	Inhibitors of DNA binding/differentiation
TIEG	TGF-β immediate early gene
IL	Interleukin
Kir	Inward recitfying potassium channels
MGP	Matrix GLA protein
MHC	Major histocompatobility class
MMP	Matrix metalloproteinase
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	Multiples sclerosis
NGF	Nerve growth factor
PAI	Plasminogen activator inhibitor
PD	Parkinson's disease
PDGF	Platelet-derived growth factor
PNS	Peripheral nervous system
SOD	Superoxide dismutase
TGF- β	Transforming growth factor β
TβR	TGF-β receptor
TNF-α	Tumor necrosis factor α
TRAF	TNF-α receptor associated factor
t-PA	Tissue plasminogen activator
VEGF	Vascular endothelial growth factor

17.1 Introduction

The isolation and characterization of transforming growth factors-β (TGF-β) by Anita Roberts has introduced a versatile extrinsic signaling molecule affecting numerous events in the life of almost each cell (Roberts and Sporn 1990; Derynck et al. 1985). Best characterized events include the regulation of cell cycle, composition of the extracellular matrix (ECM), and thereby cell migration or differentiation, as well as regulation of cell survival and death. Imbalance of TGF-β availability is therefore likely to affect tissue development, maintenance, and homeostasis. This certainly also accounts for the nervous system. Once accepted that TGF-β is also expressed in the nervous system, the functional contribution of TGF-β is now more

and more understood in numerous events in the development of the nervous system, its maintenance, and consequently also in the context of many brain diseases. This review will discuss the current knowledge of TGF-β in brain development and function and consequently in brain disorders.

Brain disorders include brain tumors, neurodegenerative disease, such as Alzheimers' disease and Parkinson's disease, insults such as ischemia and stroke, and vascular damage, as well as changes in neuronal activity, such as hyperactivity as seen in epilepsy, or neuronal depression.

17.2 TGF-β Expression in the Central Nervous System

Localisation of TGF-β isoforms in mice and rats has been performed by immunohistochemistry and in situ hybridization studies demonstrating a widespread distribution of TGF-β2 and TGF-β3 during development (Flanders et al. 1991; Pelton et al. 1991a, b; Unsicker et al. 1991). TGF-β1 is confined to meninges and choroid plexuses. During mouse development TGF-β2 and -β3 immunoreactivities become first detectable along peripheral nerves, in radial glial cells and along the central nervous system (CNS) axon tracts at embryonic age (E)12. Neuronal cell bodies become immunoreactive from E15 onwards. Most notably, TGF-β immunoreactivity is not detectable in the ventricular zone throughout the neural tube, suggesting that TGF-β may not be involved in the regulation of cell division of neural stem cells during development (Flanders et al. 1991). In contrast, on day E16, cells in the subventricular zone, subplate, and lamina I of the cortex stain positive for TGF-β. As they develop, astrocytes are also immunoreactive for TGF-β2 and -β3. In the adult nervous system both neurons and astroglia are immunoreactive for TGF-β2, -β3. Immunoreactive neuron populations include cortical layers 2, 3, and 5, hippocampus, piriform cortex, retinal ganglionic cells, hindbrain aminergic neurons, as well as spinal and hindbrain motoneurons (Unsicker et al. 1991). TGF-β1 is most prominent within the choroid plexus and meninges, it may, however, be expressed in other cells below levels of detectability. Upon lesioning, TGF-β1 may by upregulated in astrocytes as well as in neurons in vivo. TGF-β1 becomes also detectable in tissue culture, possibly mimicking a lesion-like situation. In primary neural tissue culture, treatment with all three TGF-β isoforms usually results in identical responses, suggesting that the recombinant proteins used have similar affinities for their shared receptor complex (Krieglstein and Unsicker 1994; Massague 2000).

In addition to the distribution of TGF-β within the peripheral nervous system (PNS) and CNS, its subcellular localization and mode of secretion is of importance in order to elaborate on its possible functions. Taking PC12 cells as a model to study sorting in the trans-Golgi network, Specht et al. (2003) could show that TGF-β2 may be sorted and released to a large proportion via the regulated path of secretion. Secretory vesicles provide a milieu of pH 5, which is suitable for TGF-β activation within the vesicle, enabling release of active TGF-β (Specht et al. 2003). Activity-depended release of TGF-β2 may suggest a function as a modulator for synaptic plasticity (Lacmann et al. 2007).

17.3 TGF-β in Brain Tumors

The role of TGF-β in cancer biology is complex and involves both aspects of tumor suppression (Bartholin et al. 2013; Seoane 2006) as well as tumor promotion (Wendt and Schiemann 2013; Joseph et al. 2013; Roberts and Wakefield 2003).

TGF-βs are well known for their capacity to regulate cell proliferation in a context-dependent manner. There are at least four scenarios in which regulation of cell proliferation is an important issue in nervous system development and maintenance: (a) neurogenesis, (b) proliferation of neuroblasts (neural crest cells), (c) proliferation of glial cells during development or upon lesioning, and (d) upon transformation in tumors.

Neurogenesis in the neural tube requires definite exit of the cell cycle to generate postmitotic neurons. In the past years there is increasing evidence for the role of TGF-β in developmental and adult neurogenesis (Vogel et al. 2010; Aigner and Bogdahn 2008). For some years there was indirect evidence available that neural stem cells in the neuroepithelium need to be protected from the action of TGF-β, in order to prevent premature growth retardation (Seoane et al. 2004; Hanashima et al. 2002). Seoane and coworkers have demonstrated on the basis of protein interaction analysis in human HaCaT keratinocytes that expression of the cyclin-dependent kinase (cdk) inhibitory protein 1 (p21Cip1) is regulated by TGF-β-dependent Smad complexes in combination with the Forkhead box (Fox) family member FoxO. This FoxO-Smad complex is inhibited by FoxG1, which has been shown to be essential for proliferation of telencephalic progenitor cells (Xuan et al. 1995). Indeed, FoxG1 mutants, which display reduced proliferation of telencephalic progenitor cell, premature differentiation and early depletion of the progenitor population (Xuan et al. 1995) show high levels of p21Cip1 expression in TGF-β-sensitive progenitor cells (Seoane et al. 2004). Exit from the cell cycle during terminal differentiation, as required for neurogenesis, has been described to be regulated by Ink4d and Kip1 inhibitors of cyclin-dependent kinases (Zindy et al. 1999; Cunningham and Roussel 2001). P27Kip1 has been identified as a TGF-β-dependent target gene; however, there is no evidence for a TGF-β-dependent regulation of p19Ink4d. This suggests that TGF-β may serve as an extracellular regulator to induce cell cycle arrest at the G1 phase in neural stem cells but may probably not be sufficient to regulate cell cycle exit required for terminal differentiation. In mouse hippocampal progenitor cells, TGF-β causes induction of p21Cip1 and downregulation of Cdk activators Ccnd1 and Ccnd2, leading to cell cycle exit and neuronal differentiation (Vogel et al. 2010). Along this line, TGF-β2/-β3 double knockout mice display increased cell proliferation and reduced numbers of neurons in the developing cerebral cortex and hippocampus, as the progenitor cells failed to differentiate into neurons because they did not exit cell cycle. Furthermore, TGF-β1 has been implicated as a negative modulator of adult neurogenesis (Wachs et al. 2006).

By affecting the cell cycle prior to terminal differentiation, TGF-β may, of course, regulate proliferation of neuroepithelial cells, including neuroblasts, neural crest cells, and glial progenitors (Zhang et al. 1997a; Anchan and Reh 1995).

Furthermore, TGF-β2 has been shown to regulate cell proliferation in neural crest-derived chromaffin cells (Rahhal et al. 2004) with the capacity of lifelong proliferation.

Tumors of the CNS include primitive neuroectodermal tumors, such as gliomas and medulloblastomas (Fogarty et al. 2005; Nieder et al. 2003). They derive from dividing glial cells, or neural stem and progenitor cells. Glial brain tumors are further classified using grades I–IV to express the likelihood of increased growth and malignancy. CNS tumors are characterized by rapid and infiltrative growth, angiogenesis, and immune suppression. Due to the proliferative behavior of brain cells, brain tumors show a high occurrence not only during development affecting children but also during adulthood. Particularly adult neural stem cells with the capacity to provide new neurons and glia in regions with high plasticity, following injury, or in the context of specific diseases, may escape their physiological control machinery and transform into brain cancer stem cells (reviewed in Aigner and Bogdahn 2008).

Id4 (inhibitors of DNA binding/differentiation) has been shown to serve important functions in neural stem cell differentiation and its deregulation has been implicated in glial neoplasia (Dell'Orso et al. 2010). Deregulation could occur via functional point mutations or epigenetic silencing. Martini and coworkers (2012) were able to show that epigenetic silencing of Id4 via hypermethylation resulted in reduced expression of matrix GLA protein (MGP), TGF-β1 and vascular endothelial growth factor (VEGF) which was associated with a more favorable clinical outcome. However, there are many possibilities to circumvent this effect. First, as TGF-β actions are context-dependent, the presence of certain mitogens, such as TGF-α/epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) may turn TGF-β into a growth stimulating factor (Roberts et al. 1981; Leof et al. 1986; Seoane 2006). Secondly, transformed cells may become insensitive to TGF-β due to overproduction of TGF-β or due to mutations of TGF-β receptors, their signaling components, or even their target genes responsible for G1 arrest (for example Rich et al. 1999; Lyons et al. 1990; Markowitz et al. 1995; Hahn et al. 1996; Seoane et al. 2004; Rich 2003 for review).

TGF-β´s ability to regulate ECM composition puts TGF-β at high risk in the regulation of tumor invasion and metastasis. In this context TGF-β has been shown to regulate integrin expression, for example integrin $\alpha_V\beta_3$ which has been shown to play a role in glioma propagation (Uhm et al. 1999). TGF-β has also been shown to upregulate matrix metalloprotease 2 (MMP-2) and MMP-9 expression at the cell surface (Rooprai et al. 2000) that may interact with $\alpha_V\beta_3$ integrin (for review, see Platten et al. 2001).

TGF-β is a potent immunosuppressive cytokine (Wahl and Chen 2003; Roth et al. 2012; Hau et al. 2011). Brain tumors are well known for their immunosuppressive properties allowing them to escape from the host's immune surveillance. TGF-β2 and TGF-β3 are considered master molecules that upon secretion mediate this immunosupressive environment. This immunosuppressive role has been attributed to TGF-β2, which is also the preferentially expressed isoform by many glioblastomas, grade IV gliomas (Bodmer et al. 1989; Hau et al. 2011). On this basis, TGF-β2-specific antisense gene therapy strategies have been established to make

tumor cells accessible to an effective anti-tumor immune response and counteract TGF-β-dependent tumor metastasis (Hau et al. 2009; Jachimczak et al. 1993; Lou 2004). Along this line, there is extensive research going on to identify TGF-β signaling inhibitors for cancer therapy (DaCosta et al. 2004; Yingling et al. 2004; Lahn et al. 2005).

17.4 Vascular Damage in the CNS

The blood–brain barrier (BBB) generates the specific milieu of the brain by building a tight boundary and thereby separating the components of the circulating blood from the brain. After injury or in neurologic diseases including trauma, ischemia/stroke, or Alzheimer's disease (AD), leakage of the BBB results in the entry of blood constituents into the brain (Abbott et al. 2006). Plasma proteins such as albumin, immunoglobulins, amyloid-β, and fibrinogen, and vascular cells such as erythrocytes and leucocytes, leaking into the brain have been associated with inflammation and restriction of repair (for review, see Beck and Schachtrup 2012). Schachtrup and coworkers (2010) identified TGF-β as a vascular-derived protein. Specifically, they could demonstrate that the plasma-derived protein fibrinogen acts as a carrier of latent TGF-β. Its activation is mediated via avβ6 and avβ8 integrins present on the surface of astrocytes.

Increased levels of TGF-β1 has been described in human brains during trauma, multiple sclerosis (MS), Parkinson's, AD, and stroke patients (Lippa et al. 1995). In patients suffering from severe head injury, high levels of TGF-β1 could be detected in the cerebrospinal fluid (CSF) within 1 day after injury (Csuka et al. 1999; Morganti-Kossmann et al. 1999). These observations strongly suggest that lesion-induced, vascular-derived TGF-β contributes to the corresponding degeneration and regeneration processes (Beck and Schachtrup 2012).

TGF-β has been profoundly investigated for its role in orchestrating the response to brain lesions (for review, see Flanders et al. 1998). With regard to astrocytes, this includes regulation of astrocytic growth, astroglial scar formation, and anti-inflammatory responses. In most contexts studied, TGF-β inhibits the growth of astrocytes (Flanders et al. 1993; Hunter et al. 1993). Most importantly, TGF-β counteracts mitogenic signals by astroglial mitogens such as fibroblast growth factor-2 (FGF-2) or PDGF. However, effects may vary depending on astrocyte culture conditions in vitro or may be brain region-dependent in vivo (Labourdette et al. 1990; Johns et al. 1992). TGF-β may also affect cell adhesion, migration, and ECM production by astrocytes, all being important steps in the cascade of shaping the reactive astrocyte phenotype. TGF-β-treated astrocytes show a slight increase in actin content, the appearance of actin stress fibers, a slight increase in the glial fibrillary acidic protein (GFAP), and an increased production of laminin and fibronectin (cf. Baghdassarian et al. 1993). Thus, treatment of cerebral wounds with anti-TGF-β2 antibodies was shown to lead to a marked reduction of glial scarring (Logan et al. 1999). Many effects of TGF-β on astroglia are anti-inflammatory

and immunosuppressive, as TGF-β modulates the expression of important cytokines involved in CNS immune reactions. These include upregulation of interleukin-6 (IL-6) and nerve growth factor (NGF) (Spittau et al. 2012; Aderka et al. 1989; Lindholm et al. 1992), blocking interferon-γ mediated upregulation of major histocompatibility class (MHC) II (Dong et al. 2001), and the tumor necrosis factor α (TNF-α) and interleukin-1 β (IL-1 β)-mediated upregulation of intercellular adhesion molecule-1 (Shrikant et al. 1996).

17.5 TGF-β in Neuronal Survival and Death

TGF-β has been shown to promote survival of several neuronal populations in vitro (Krieglstein et al. 1995; Poulsen et al. 1994; Martinou et al. 1990). However, it is now well established that TGF-β may modulate the neurotrophic capacities of numerous growth factors including neurotrophins, such as NGF, brain-derived neurotrophic factor (BDNF), as well as ciliary neurotrophic factor (CNTF) (Krieglstein and Unsicker 1996) and, most importantly, glial cell line-derived neurotrophic factor (GDNF; Krieglstein et al. 1998b). GDNF was shown to crucially depend on TGF-β to exert its neurotrophic activities on peripheral as well as mesencephalic dopaminergic neurons in vitro. In vivo, GDNF's neuroprotective effect on target-deprived pre-ganglionic sympathetic neurons, as well as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned nigrostriatal dopaminergic neurons, also depends on the presence of TGF-β (Schober et al. 1999, 2007). GDNF/TGF-β cooperativity on chick ciliary ganglionic neurons has now been characterized in detail, whereby TGF-β is required for appropriate GDNF receptor (GFRα1) recruitment to the plasma membrane (Peterziel et al. 2002). Interestingly, TGF-β does not cooperate with Neurturin, a closely related factor to GDNF, and does not promote the recruitment of GFRα2 to the plasma membrane, suggesting high specificity in TGF-β/GDNF cooperativity (Peterziel et al. 2007).

Depending on the cellular context, TGF-β has also been shown to regulate ontogenetic neuron death. Upon immunoneutralization of all TGF-β isoforms in ovo (E6–E10), ontogenetic cell death of chick parasympathetic ciliary ganglionic neurons, sensory dorsal root ganglionic neurons as well as lumbar spinal motoneurons could be prevented (Krieglstein et al. 2000). Similarly, TGF-β regulates ontogenetic morphogenetic cell death in the developing retina of chick and mouse embryos (Dünker et al. 2001; Dunker and Krieglstein 2003). Another classical model for morphogenetic cell death during embryogenesis represents the removal of interdigital tissue to form individual fingers. Similarly, double deletion of TGF-β2 and -β3 in the mouse resulted in lack of cell death (Dunker et al. 2002). Furthermore, induced neuron death following embryonic limb bud ablation in chick embryos resulted in a significant neuroprotection upon immunoneutralization of TGF-β (Krieglstein et al. 2000). Together, these data suggest that TGF-β is a key regulator of ontogenetic cell death in vivo. Mechanistically, we recently identified that TGF-β-induced apoptosis in oligodendroglial progenitor cells (OLI-neu; Schuster et al. 2002) is characterized

by downregulation of Bcl-xl. Furthermore, Fractin is produced as a caspase-specific cleavage product in oligodendroglial cells during TGF-β-mediated apoptosis, whereby Fractin binding to Bcl-xl induced downregulation of Bcl-xl protein levels (Schulz et al. 2009). Sorrentino and collaborators (2008) were able to show that the intracellular apoptotic cascade can be initiated via the type I receptor of TGF-β (TβR-I) and receptor-engaged TNF-α receptor associated factor 6 (TRAF6). Although TGF-β-induced apoptosis and underlying signaling pathways have been well characterized in many cells types, little is known about TGF-β-induced apoptosis in neurons (Schuster and Krieglstein 2002; Sanchez-Capelo 2005).

17.6 Cerebral Ischemia

Cerebral ischemia is caused by either a blood clot occluding a blood vessel in the brain (focal ischenia) or a more general reduction in brain blood flow (global ischemia) leading to insufficient blood flow and reduced oxygen levels in the respective brain areas, thus leading to death of brain tissue. Neuronal cell death may occur as necrosis or apoptosis. Thrombolysis is the approved treatment of stroke. TGF-β1 expressed at low levels in adult brain is rapidly upregulated following insults such as cerebral ischemia, excitatory injury, or traumatic brain injury (Klempt et al. 1992; Knuckey et al. 1996; Yamashita et al. 1999; Morganti-Kossmann et al. 1999; Zhu et al. 2000; Boche et al. 2003; Krieglstein 2006; Pál et al. 2012). TGF-β1 upregulation was observed primarily in microglial cells and in astrocytes, while TGF-β2 upregulation was seen in neurons. TGF-β3 was not upregulated; however, the levels of both TGF-β2 and TGF-β3 decreased subsequently. These data suggest a distinct spatiotemporal requirement of TGF-β isoforms action during cerebral ischemia (Pál et al. 2012). As TGF-β is a good candidate to organize the response of neurons to degeneration as well as mediating anti-inflammatory reactions, its neuroprotective potential has been widely analyzed (for review, see Flanders et al. 1998; Böttner et al. 2000; Dobolyi et al. 2012). Specifically, TGF-β1 applied either as recombinant protein or by adenoviral-based overexpression has been shown to reduce infarct size after focal cerebral ischemia and to prevent hippocampal neuronal damage after global ischemia (Gross et al. 1993; Prehn et al. 1993; Zhu et al. 2002; for review, see Buisson et al. 2003; Dhandapani and Brann 2003). Furthermore, TGF-β may also mediate tolerance of ischemic preconditioning towards subsequent ischemic insult (Boche et al. 2003). The molecular mechanism(s) by which TGF-β protects neurons from ischemic cell death relies on a signaling crosstalk between neurons and astrocytes (Prehn et al. 1994; Docagne et al. 1999) and involves the maintenance of Ca^{2+} homeostasis, modulation of the t-plasminogen activator (tPA)/plasminogen activator inhibitor (PAI-1) axis, as well as inhibition of pro-apoptotic pathways, such as Bad and caspase-3 (Zhu et al. 2001, 2002) and upregulation of anti-apoptotic proteins such as Bcl-2 (Prehn et al. 1994). An additional TGF-β-dependent anti-apoptotic pathways involving NF-κB activation has been described (Zhu et al. 2004). This pathway seems to be downstream of ALK1 (activin receptor-like kinase

1), an alternative TGF-β type I receptor first described on endothelial cells, which has been shown to be upregulated in neurons in an injury-dependent manner (Konig et al. 2005). Injury-dependent upregulation of ALK1, with signaling preference towards Smad1, may also explain numerous opposing effects of TGF-β in brain development and lesions.

17.7 Alzheimer's Disease

Alzheimer's disease (AD) is a degenerative brain syndrome characterized by a progressive decline in learning and memory, thinking, language, judgment, and other higher brain functions. Currently about 18 million people worldwide suffer from AD and it is estimated that in 2025, 34 million will be affected. The statistical risk of occurrence is 1.4 % at the age of 60 and doubles every 5 years thereafter (WHO; Huang and Mucke 2012).

AD is characterized by considerable brain shrinkage resulting in a large loss of brain weight and volume. The extent of brain volume loss suggests more general mechanisms such as shrinkage and loss of neuronal processes, including degeneration of specific neuron populations (Huang and Mucke 2012). Along this line, aberrant neuronal network activity, dysfunction, and loss of synapses may describe the cognitive decline in AD.

TGF-β has been implicated in the regulation of neurite outgrowth, transmitter synthesis as well as synapse formation (Krieglstein et al. 2011). TGF-β has been reported to cause neurite sprouting and elongation of hippocampal axons as well as promoting re-elongation of injured axons of hippocampal neurons in vitro (Ishihara et al. 1994; Abe et al. 1996). In the mouse neocortex, TGF-β can direct neuronal polarity by initiation of axon formation and neuronal migration via site-specific phosphorylation of the polarity protein Par6 (Yi et al. 2010). Extracellular signaling factors such as Wnt and TGF-βs are recognized as target-derived signals in synaptogenesis (Salinas 2005; Packard et al. 2003). In chick ciliary ganglionic neurons, developmental expression of K_{Ca} channels coincides with synaptogenesis. Dryer and coworkers have shown that target-derived TGF-β1 regulates the developmental expression of Ca^{2+}-activated K^+ currents in vitro and in vivo (Cameron et al. 1999). The acute effect of TGF-β1 relies on the translocation of K_{Ca} channels from intracellular stores to the plasma membrane involving signaling via the Ras GTPase, extracellular regulated kinases (Erk), and phosphoinositide 4′ (PI4) kinase (for review, see Dryer et al. 2003). In conclusion, TGF-β may well be suited to modulate synaptic plasticity and cognition (for review, see Krieglstein et al. 2011).

The pathogenesis of AD is focusing on amyloid β (Aβ) peptides, the main constituent in plaques, derived from amyloid precursor protein (APP) upon proteolylic cleavage (De Strooper et al. 2010; Bertram et al. 2010). There are several lines of evidence suggesting that TGF-β1 may contribute to the pathology of Alzheimer's disease, particularly through promoting Aβ precursor expression and Aβ deposition (Burton et al. 2002; Wyss-Coray et al. 1997a, b; Flanders et al. 1995;

van der Wal et al. 1993). Mice overexpressing TGF-β1 in astrocytes develop AD-like vascular and meningeal abnormalities with age (Gaertner et al. 2005). These chronic alterations could be correlated with reduced brain tissue perfusion, leading to an increased amount of fibrillar and soluble Aβ peptides. However, in brain parenchyma, astroglial TGF-β1 expression leads to a reduction of overall Aβ as well as decreased numbers of dystrophic neurites (Wyss-Coray et al. 2001). The reduced plaque burden in brain parenchyma is thought to depend on TGF-β-dependent microglial activation and microglial Aβ-clearance. Furthermore, a genetic association study of three polymorphisms of the human TGF-β1 gene with AD suggests that there is no correlation between TGF-β1 and AD on the basis of TGF-β1 gene variability (Araria-Goumidi et al. 2002).

Most recently, bioactive TGF-β has been shown to be associated with lipoproteins, specifically those containing ApoE3, but not ApoE4 (Tesseur et al. 2009). Association of TGF-β1 with lipoproteins may facilitate its diffusion and signaling and possibly also other biological functions of TGF-β1. This observation is of particular interest in this context as ApoE4 has been genetically linked with late-onset AD (Bertram et al. 2010). As TGF-β1 is a neuroprotective agent and may be beneficial in the AD condition, for example through reduction of plaque burden, the preferential binding of TGF-β1 to ApoE3 versus ApoE4 may put ApoE4 carriers on higher risk for developing AD (Tesseur et al. 2009).

Several lines of evidence suggest impairment of TGF-β-activated Smad signaling, with ectopic localization of phosphorylated Smad2/3 within amyloid plaques and neurofibrillary tangles (Lee et al. 2006; Tesseur et al. 2006; Ueberham et al. 2006; Chalmers and Love 2007a, b). Furthermore, AD patients have been shown to have reduced plasma levels of TGF-β1 (Mocali et al. 2004; Juraskova et al. 2010) as well as reduced neuronal expression of the TGF-β type II receptor (TβRII) (Tesseur et al. 2006). Lack of TβRII signaling via neuronal expression of kinase-deficient TβRII in AD transgenic mice promoted Aβ deposition and loss of dendrites (Tesseur et al. 2006), while Aβ may downregulate expression of TβRII via induction of miR-106b (Wang et al. 2010). Finally, injection of synthetic Aβ in combination with blocking TGF-β signaling via application of the TβRI kinase inhibitor SB431542 significantly increased the vulnerability of hippocampal neurons to Aβ, leading to neuronal degeneration (Caraci et al. 2008).

Together, as TGF-β1 signaling is beneficial in the AD environment, rescuing TGF-β1 levels and TGF-β signaling may represent a new strategy for neuroprotection in AD (Caraci et al. 2012).

17.8 Parkinson's Disease

Parkinson's disease (PD) is a neurodegenerative disease characterized by a progressive loss of nigrostriatal neurons and in consequence by marked reduction of striatal dopamine resulting in impaired voluntary movement (for review, see Braak et al. 2004; Dunnett and Björklund 1999). In addition to the loss of neurons, a further

morphologic hallmark of PD is the presence of Lewy bodies and Lewy neurites (Forno 1996). The formation of these proteinaceous inclusions involves interaction of several proteins, including α-synuclein (Spillantini et al. 1998). The etiology of PD is likely due to combinations of environmental and genetic factors (for review, see, for example, Valente et al. 2012; Gasser et al. 2011).

TGF-β2 and TGF-β3 are expressed in adult nigral dopaminergic neurons (Unsicker et al. 1991), and TGF-β1 and -β2 were elevated in biopsies of PD patients (Nagatsu et al. 2000). TGF-βs have been shown to promote midbrain dopaminergic neuron survival in vitro and in vivo (Krieglstein and Unsicker 1994; Poulsen et al. 1994; Roussa et al. 2004), as well as protection against 1-methyl-4-phenylpyridinium (MPP+) intoxication (Krieglstein et al. 1995; Roussa et al. 2009). Most importantly, TGF-β cooperates with GDNF to promote dopaminergic neuron survival (Krieglstein et al. 1998b). GDNF is well known as a potential therapeutic agent coping with PD (for review, see Björklund and Lindvall 2000). However, also in vivo GDNF-dependent neuroprotective effects are based on the cooperativity with TGF-β, as shown in the MPTP-mouse model, an animal model of PD (Schober et al. 2007). This neuroprotective strategy has already been used incidentally by grafting extra-adrenal chromaffin cells obtained from Zuckerkandl's organ in parkinsonian rats (6-hydroxydopamine model). The behavioral improvements of parkinsonia deficts were in addition to the supply of catecholamines attributed to the release of the survival promoting proteins GDNF and TGF-β1 (Fernandez-Espejo et al. 2005).

Most recently, a new animal model for PD has been introduced. Viral transformation of rats with α-synuclein showed a slow progression of nigral dopaminergic neurons (Ulusoy et al. 2010). Most interestingly, GDNF, for long considered as the gold standard in neurotrophic-based neuroprotection of PD, is not able to rescue α-synuclein-mediated degeneration of dopaminergic neurons (Decressac et al. 2011).

17.9 Epilepsy

Epilepsy is a common neurological disorder affecting 0.5–2 % of the population worldwide. Epilepsy is characterized by seizures resulting from abnormal neuronal activity. So far, there is no cure known for the disease. The mechanisms leading to the disease are only poorly understood. However, epilepsy is often seen following brain trauma, ischemic or infectious brain injury, or drug and alcohol misuse. These conditions may be accompanied by vascular damage and leakage of the blood–brain barrier (see above).

Cacheaux et al. (2009) have identified the involvement of TGF-β signaling in epileptogenesis. The group has previously demonstrated that serum albumin causes epileptic field potentials when exposed to brain slices in vitro (Ivens et al. 2007). Serum albumin was taken up by astrocytes leading to down regulation of inward-rectifying potassium (Kir 4.1) channels, resulting in reduced buffering of extracellular potassium in neuronal hyperexcitability and epileptiform activity. As the albumin uptake was shown to occur in a TGF-β receptor-mediated mechanism,

blocking of the TβR in vivo reduced the likelihood of epiletogenesis in albumin exposed brains (Ivens et al. 2007). In follow-up experiments, the group was able to show that TGF-β1-mediated signaling is sufficient to induce epileptoforming activity (Cacheaux et al. 2009). These data strongly link the TGF-β pathway with epileptogenesis and identify the TGF-β pathway as a therapeutic target for the prevention of injury-induced epilepsy (Friedman 2011).

17.10 Depression

Major depressive disorder is a mental disorder characterized by low mood, low self-esteem, and reduced interest in enjoying pleasure. There is no clinical test for depression. Patients are treated with antidepressant drugs, which improves their mental condition after several weeks of treatment. Most antidepressant medications directly or indirectly increase the levels of one or more of the monoamines, such as serotonin, noradrenaline, and dopamine, in the synaptic cleft between neurons in the brain, suggesting that depression may be the consequence of reduced synaptic activity. In addition to neurotransmitters, also neuropeptides and neurotrophic factors such as BDNF and TGF-β have been shown to be released in an activity-dependent manner (Thoenen 1995; Specht et al. 2003; Lacmann et al. 2007). Notably, KCl stimulation caused Smad translocation into the nucleus and induced TGF-β-inducible early gene (Tieg1) expression, demonstrating that activity-dependent released TGF-β may exert autocrine actions and thereby activate the TGF-β-dependent signaling pathway (Lacmann et al. 2007). These results suggest an activity-dependent release and gene transcription of TGF-β in mouse hippocampal neurons in vitro as well as subsequent autocrine functions of the released TGF-β within the hippocampal network. TGF-β is also known to have a prominent role in long-term synaptic facilitation in isolated Aplysia ganglia (Zhang et al. 1997b). Within minutes, TGF-β1 stimulated MAPK-dependent phosphorylation of synapsin, which appeared to modulate synapsin distribution, and resulted in a reduced magnitude of synaptic depression (Chin et al. 2002). Most recently, Fukushima and coworkers (2007) were able to show that TGF-β modulates synaptic efficacy and plasticity in dissociated rat hippocampal neurons. Together, increasing evidence suggests that TGF-β may be involved in synaptogenesis, modulation of synaptic transmission, and synaptic plasticity.

The delayed effects of antidepressants are thought to depend on indirect mechanisms, including the regulation of gene expression, for example antidepressant-induced upregulation of BDNF signaling, which then in turn promotes adaptive neuronal plasticity through effects on gene expression. Wibrand and coworkers (2006) identified five genes (Neuritin, Narp, Tieg1, Carp, and Arl4d) that are co-upregulated with Arc during BDNF-LTP. Tieg1 is a TGF-β-dependent immediate early gene that has also been shown to be upregulated in hippocampal neurons by TGF-β (Lacmann et al. 2007). As TGF-β has been shown to cooperate with BDNF in several scenarios, it may be quite likely that BDNF and TGF-β are the key players in antidepressant-mediated restoration of neuronal plasticity in patients suffering from major depressive disorders.

17.11 Motoneuron Disease

Amyotrophic lateral sclerosis (ALS) is a fatal disease, leading to paralysis and death. It is characterized by loss of motoneurons. Some ALS cases are due to mutation of the superoxide dismutase 1 (SOD1). TGF-β is a prominent motoneuron survival factor (McLennan and Koishi 2002; Martinou et al. 1990). Using SOD1 knockout mice as a model for ALS application of TGF-β2 caused a rapid and marked but transient improvement in the motoric performance of the mice (Day et al. 2005). In the past years, all components of the TGF-β signaling system have been localized in the presynaptic terminal of the neuromuscular junction, whereby TGF-β ligands are synthesized and localized on the postsynaptic side (McLennan and Koishi 2002; Toepfer et al. 1999). Furthermore, it has been shown that TGF-β2 alters the characteristics of the neuromuscular junction by regulating presynaptic quantal size (Fong et al. 2010).

Appropriate myelination is an important aspect of neuronal activity. Oligodendroglial cells are the myelinating cells of the CNS and Schwann cells of the PNS. Oligodendrocytes arise from a bipotential progenitor cell, the O2A progenitor. TGF-β restricts their PDGF-driven proliferation and induces oligodendroglial differentiation (McKinnon et al. 1993) but may also induce apoptosis (Schuster et al. 2002). In the PNS, TGF-β mediates developmental cell death of Schwann cells (Parkinson et al. 2001) and blocks Schwann cell myelination and expression of myelin-related proteins (Awatramani et al. 2002 and references therein). However, in adult mice TGF-β seems to stabilize compact myelin, as TGF-β1-null mice have grossly abnormal myelin (Day et al. 2003). Ski, a repressor of Smad-mediated TGF-β signaling controls Schwann cell proliferation and myelination, whereas absence of Ski abolished the formation of peripheral myelin, and myelinating Schwann cells upregulate Ski in development as well as during remyelination upon injury (Bonnon and Atanasoski 2012; Atanasoski et al. 2004).

17.12 Conclusions

TGF-β is a multifunctional and versatile molecule, effecting development, adult maintenance as well as aging of the brain. Although the prototype of a superfamily TGF-β is acting in the nervous system in a highly specific manner. In the context of brain tumors TGF-β is acting very much the way it as expected to by regulating cell cycle, adhesion, and immunosuppression. However, in all other disease scenarios TGF-β action is much more versatile reaching from orchestrating astrocyte and microglia activation to regulating growth factor responsiveness, uptake and release mechanisms, activity-dependent gene expression, and nerve myelination. TGF-β responsiveness may be mediated via alternative TβR-1 usage and may be blocked via the miRNA-dependent downregulation of TβR-II. A new and fascinating aspect is also evident from the modes of TGF-β delivery within the body through transport via lipoproteins or via fibrinogen. In conclusion, the role of TGF-β within brain

disorders, either as cause or as key molecule orchestrating responses is only at the beginning of its understanding. Major open issues regard the specific action of individual TGF-β isoform, the context-specificity, the role of TGF-β activation to regulate TGF-β function and complexity of TGF-β signaling and crosstalk. This knowledge will then be also helpful to explain opposing actions of TGF-β such as promotion of survival/protection as to induction of cell death.

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Chapter 18
TGF-β and Metabolic Homeostasis

Grace Bennett and Sushil G. Rane

Abstract Metabolic homeostasis is achieved via a concerted and integrative action of various organ systems. The harmonious functionality of the collaborative organs maintains tight regulation of glucose levels. This intricate balance of glucose production and utilization maintains whole body glucose homeostasis and energy balance. In disease states, such as diabetes and obesity, dysfunction in one or more organ systems disturbs the metabolic homeostasis and propagates disease pathology. Understanding the avenues that dictate metabolic homeostasis and the pathways that disrupt this harmony are thus of great medical significance. We propose that the TGF-β signaling network plays an integral role in metabolic homeostasis by virtue of its actions on several organ systems that constitute the metabolic machinery. TGF-β levels are elevated in metabolic disease, which supports the utility of therapeutics aimed at targeting the TGF-β pathway to combat these diseases. Considering the complexity of the TGF-β signaling network, a rational approach is vital to designing anti-TGF-β modalities to combat metabolic diseases.

Keywords Adipocytes • Adipose tissue • Diabetes • Homeostasis • Insulin • Metabolism • Mitochondria • Obesity • Pancreas • Smad3 • TGF-β • β-cells

18.1 Metabolic Homeostasis

For living organisms to maintain a state of metabolic homeostasis there must be a steady state of nutrient and energy intake and its utilization. Macro- (carbohydrate, lipid and protein) as well as micro-nutrients (vitamins, minerals, and trace elements) are the components of the diet which provide the energy, molecular building blocks,

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co-enzymes, and co-factors needed to maintain properly functioning metabolic processes of nutrient digestion, absorption, utilization, and excretion. There are specific "metabolic tissues" that are primarily responsible for the breakdown and utilization of these nutrients. Because of their critical importance in supporting the life of an organism, the normal physiology of these tissues is well characterized, in particular the insulin responsive tissues (adipose tissue, liver, skeletal muscle) and the sole insulin producing tissue, the pancreas. However, we have gained insight into the importance of these tissues in metabolic homeostasis through their pathological contexts as well (Biddinger and Kahn 2006). In particular, the obese state, along with its metabolic comorbidities, has shed light on the integrated role of these organ systems, although this has also created the problem of determining which of these tissues is the primary site responsible for disease states such as type II diabetes (Yang and Trevillyan 2008; Olefsky and Glass 2010).

The adaptation of a stomach and adipose tissue has made it possible to eat in the meal format, where, as opposed to grazing, an organism consumes food in discrete settings until they reach a level of "fullness," stop eating while still fueling their cells to carry out their basic physiological processes due to adequate nutrient stores within the body.

After a meal, carbohydrates are broken down to glucose molecules and are either utilized by the intestine or liver (the first organs they encounter) or are sent to all other tissues where they can be utilized for the synthesis of ATP after conversion to pyruvate via glycolysis. In order for glucose to gain entry into insulin sensitive tissues, i.e., adipose tissue and skeletal muscle, glucose needs insulin, an endogenously secreted peptide hormone from the β-cells of the pancreas. Insulin binds its receptor on the cell surface of insulin responsive tissues. The receptor, being a receptor tyrosine kinase, autophosphorylates and begins a signal transduction cascade that involves insulin receptor substrates (IRS), phosphatidylinositol 3 phosphate (PI3K), and Akt. Activation of this pathway by phosphorylation releases premade vesicles containing glucose transporter 4 (GLUT4) to the cell surface thereby allowing for the entry of glucose into the cell (Chang et al. 2004). This glucose is used for the basic cellular needs, and what remains is converted to triacylglycerol through the process of de novo lipogenesis occurring primarily in the liver and adipose tissue.

Fatty acids consumed in a meal undergo a different metabolic process than carbohydrates, but, in part share the same fate being utilized for immediate energetic needs or stored in liver as glycogen or in adipose as triacylglycerol for later use. Fatty acids are typically found in a meal as triglycerides, which cannot enter the intestinal cells. In the small intestines, these triacylglycerols are bound by pancreatic lipases that break them down into component fatty acids and monoacylglycerols. However, these lipases are only able to work at the surface of these fats. Emulsification by bile salts created in the liver and released from the gall bladder creates more surface area for these lipases to work ultimately allowing for their entry in enterocytes of the small intestines to be reformed into triglycerides and packaged into chylomicrons before release into the lymphatic circulation. Unlike glucose, fatty acids do not need insulin to gain entry to adipocytes. Rather,

lipoprotein lipase on the cell surface releases fatty acids into the cell where they can be stored as triacylglycerol in a lipid droplet or oxidized.

18.1.1 Adipose Tissue

In obesity, chronic over nutrition leads to the need to store excess amounts of energy, manifest as increased storage of triacylglycerols in adipose tissue, with "spillover" resulting in ectopic fat deposition in liver, skeletal muscle, and pancreas (Cusi 2010; Unger and Scherer 2010). Adipocytes respond to this need to store excess energy in a depot-specific manner: subcutaneous adipocytes (under the skin) grow by hyperplasia (i.e., increasing the amount of adipocytes able to store triacylglycerols via proliferation of progenitor cell pools) (Ibrahim 2010). However, adipocytes of the visceral cavity (intra-abdominal adipose tissue) accommodate excess lipid by physically expanding the size of each cell (i.e., hypertrophy) (Ibrahim 2010). Hypertrophic adipocyte growth is considered a cellular stress in terms of both sheer and mechanical stress as the surface area to volume ratio changes, but also with regard to disruption of the normal physiological processes occurring within the cellular machinery (Bays et al. 2008; Cusi 2010; Ghorbani et al. 1997; Nishimura et al. 2007; Khan et al. 2009; Kim et al. 2007b). For example, hypertrophic adipocytes are susceptible to ER-stress (Guo et al. 2007), decreased secretion of insulin sensitizing agents such as adiponectin (Greenberg and Obin 2006), decreased insulin sensitivity (Biddinger and Kahn 2006), increased secretion of inflammatory mediators (Gutierrez et al. 2009; Halberg et al. 2009; Jager et al. 2007; Kang et al. 2008; Karalis et al. 2009; Kim et al. 2007a; Lacasa et al. 2007; Lumeng and Saltiel 2011; McGillicuddy et al. 2011; Odegaard et al. 2007; Olefsky and Glass 2010; Perfield et al. 2010; Shi et al. 2006; Shoelson et al. 2006), increased lipolysis and increased cell death (Cinti et al. 2005; Greenberg and Obin 2008; Greenberg and Obin 2006). The latter three processes initiate a vicious cycle that exacerbates the negative impact of adipocyte hypertrophy on metabolism as cell death triggers production of inflammatory mediators and releases free fatty acids into the local environment. Moreover, inflammatory mediators promote increased lipolysis and insulin resistance by blocking insulin receptor signaling through inhibitory phosphorylation of the insulin receptor, thereby blocking the anti-lipolytic actions of insulin. Increased release of free fatty acids via lipolysis promotes the secretion of inflammatory mediators through free-fatty acid activation of Toll-like receptor 4 (TLR4), resulting in transcription of inflammatory mediators (TNFα, IL-6, etc.), thereby propagating the signal which promotes insulin resistance and leads to the recruitment of many immune cell populations (Fain 2006; Weisberg et al. 2003; Zeyda and Stulnig 2007; Suganami et al. 2007; Shaul et al. 2010; Strissel et al. 2010; Strissel et al. 2007). All of the aforementioned processes have led to the hypothesis that insult to adipose tissue is the primary lesion leading to systemic hyperglycemia, hyperinsulinemia, insulin resistance, and diabetes mellitus type II.

18.1.2 Liver

As described above, it is clear that the liver plays a vital role in metabolism. The liver is the only organ in the body that can produce glucose and therefore plays an extremely important role in metabolic homeostasis during times of fasting or between meals. In times of fasting, the drop in blood glucose concentration triggers the liver to respond by utilizing glycogen stores to produce glucose for release into circulation, to maintain the blood glucose concentration within the tight range of 80–100 mg/dL. After a meal, the liver gets the first look at nutrients comprising what was consumed. Some carbohydrates are utilized to replace any glycogen stores that may have been depleted in between meals in a process regulated by the anabolic actions of insulin, before the rest are sent to the peripheral tissues for maintenance of blood glucose concentration, metabolic use or if a surplus exists, eventual conversion to triacylglycerol for storage in adipose tissue. Pathologic features of obesity that impact the ability of the liver to function normally are hepatic steatosis as well as inflammation. Hepatic steatosis can occur endogenously by increased synthesis of triacylglycerol from excess carbohydrates as well as from ectopic lipid deposition (i.e., lipid spillover from saturated adipose tissue) (Cusi 2010; Unger et al. 2010; Unger and Scherer 2010). This creates controversy when trying to ascribe the pathogenic blame of metabolic dysregulation. For example, while it is recognized that adipose tissue becomes temporarily saturated with lipid before it is able to expand its cellular pools, spilling lipids over into the liver, the liver can also produce endogenous fatty acids from de novo lipogenesis. This makes it difficult to determine the source and kinetics of ectopic fat found in liver. Be that as it may this lipid, regardless of the source, induces local inflammation within the liver and recruitment of Kupffer cells followed by the same inflammation induced insulin resistance in liver as in adipose tissue (Saberi et al. 2009). However, unlike adipose tissue, when the liver is insulin resistant, it can no longer respond to the signal from insulin after a meal to stop producing glucose and store glycogen. Therefore, insulin resistance in the liver is a primary cause of hyperglycemia, making some argue that the liver's contribution to metabolic dysregulation is more important than that of adipose tissue.

18.1.3 β-Cells

β-cells comprise about 65–80 % of the islets of Langerhans within the pancreas, where they make and store insulin. As mentioned, the liver is a strong determinant of basal blood glucose concentration. However, when blood glucose levels increase rapidly, as they do after a meal, for example, the pancreas senses this and β-cells release two waves of insulin. The first wave releases the premade insulin rapidly, within minutes, while, the second wave occurs about 30 min post-prandially.

The β-cells also release C-peptide and Amylin, and the pancreas in general secretes many endocrine and exocrine products. Here, we will focus on the role of β-cells in insulin/glucose homeostasis. β-cell destruction by auto-antibodies leads to diabetes mellitus type I, while "β-cell fatigue" is the final step in the progression from obesity, tissue-specific insulin resistance, hyperglycemia, whole-body insulin resistance, and ultimately diabetes mellitus type II (Biddinger and Kahn 2006). The former typically occurs in adolescents and young adults, while the latter is a much more chronic process which takes years to develop. However, in the current obesigenic environment, increasing numbers of adolescents are being diagnosed as type II diabetics.

18.1.4 Skeletal Muscle

Muscle is able to aid in gluconeogenesis via transfer of amino acid substrates to the liver for conversion into carbon skeletons for glucose production; however, the large size of skeletal muscle and its responsiveness to insulin are what create the major role for this tissue in glucose homeostasis as the predominant site of glucose utilization (Thiebaud et al. 1982). As with liver and adipose tissue, inflammation and free fatty acids interfere with the function of insulin receptor signaling in skeletal muscle, leading to decreased glucose uptake and hyperglycemia (Kennedy et al. 2009; Nieto-Vazquez et al. 2008; Senn 2006; Weigert et al. 2004). It has been dogma for some time that skeletal muscle insulin resistance is the most critical component to developing whole-body insulin resistance and diabetes mellitus type II (DeFronzo and Tripathy 2009); however as mentioned above, the contributions of multiple tissue types mean that it is not difficult to see how intimately intertwined adipose tissue, muscle, β-cells, and liver are in maintaining metabolic regulation. Euglycemic/hyperinsulinemic clamp studies have made it possible to tease apart the relative contribution of liver and skeletal muscle to hyperglycemia/insulin resistance phenotype in human subjects as well as experimental settings that utilize animal models (Borai et al. 2011). However, the timing of both adipose tissue's deleterious contributions as well as failure of β-cells to compensate by increasing production of insulin remains to be elucidated.

Bariatric surgeries, in which parts of the intestinal tract are removed or bypassed, have shed light onto the importance of the intestinal-brain axis and related hormones in metabolic homeostasis, but as this is beyond the scope of this chapter, we point you to the work of Andreelli et al. for a more detailed review (Andreelli et al. 2009).

In summary, metabolic homeostasis is the ability to maintain body weight and physiological processes via mechanisms which balance energy intake, expenditure, and storage. These processes involve many tissues and organ systems, and dysregulation of one part can affect the whole organism leading to metabolic complications, weight gain, and/or disease.

18.2 Role of TGF-β Signaling in Metabolic Tissues

The ability to properly specify, differentiate, and/or proliferate in the early developmental stages of metabolic tissues is of critical importance to metabolic homeostasis. TGF-β and their family members: BMPs, activins, and GDFs have all been well characterized in their roles in determination of cellular fate, in particular, in early development and stem cell biology (James et al. 2005; Chng et al. 2011; Wu et al. 2008; Watabe and Miyazono 2009; Takenaga et al. 2007; Willems and Leyns 2008; Kitisin et al. 2007; Varga and Wrana 2005; Mishra et al. 2005). Here, we review the importance of the TGF-β signaling pathway in metabolic homeostasis based on its important actions on two organs—the adipose tissue and the pancreatic β-cell.

18.2.1 Role of TGF-β Signaling in Adipose Tissue Biology

Obesity is principally characterized by fat accumulation in the adipose tissue depots (Gesta et al. 2007; Park et al. 2008). Mammalian adipose tissue has been traditionally classified as white adipose tissue (WAT) and brown adipose tissue (BAT). WAT functions predominantly as an energy storage depot characterized by large unilocular lipid droplets and serves as a prominent endocrine organ producing hormones that regulate feeding and satiety (Rosen and Spiegelman 2006). In contrast, BAT is involved in energy dissipation and is characterized by multilocular cells that harbor densely packed mitochondria and expression of uncoupling protein-1 (UCP1) (Cannon and Nedergaard 2004, 2011). Copious amount of brown fat exists in rodents, hibernating animals, and human infants—and until recently brown fat was considered to be nonexistent in adult humans. Recent findings that metabolically active brown fat exists in humans (Nedergaard et al. 2007; Cypess et al. 2009; van Marken Lichtenbelt et al. 2009; Virtanen et al. 2009), and that levels of brown fat activity might vary with age and adiposity, have re-stimulated interest concerning the therapeutic potential of augmenting brown fat to combat metabolic diseases such as diabetes and obesity (Nedergaard and Cannon 2010; Enerback 2010). Interestingly, lineage tracing data supports the notion that brown fat shares its developmental origin with muscle, and not white fat as it was long presumed (Atit et al. 2006; Timmons et al. 2007). Further, the transcription factor PRDM16 determines the fate of $Myf5^{+}$-precursor cells towards the brown fat lineage (Seale et al. 2009; Seale et al. 2008).

18.2.2 Brown Adipocyte Induction in Traditional WAT Depots

Brown adipocytes are found interspersed within the WAT depots, in response to chemical or hormonal stimulation, cold exposure, or defined genetic manipulation

(Langin 2009; Lefterova and Lazar 2009; Frontini and Cinti 2010). Expression of the brown adipocyte marker UCP1 is increased in the multiple adipose tissue depots upon exposure to cold (Loncar 1991) or upon treatment with a β3-adrenoceptor agonist, CL 316,243 (Cousin et al. 1992). In agreement, β3-adrenoceptor knockout mice have reduced numbers of brown adipocytes in white fat upon cold exposure (Jimenez et al. 2003), whereas transgenic mice overexpressing the β1-adrenergic receptor in adipose tissue exhibit abundant appearance of brown fat cells in WAT and are resistant to obesity (Soloveva et al. 1997). Further, chronic treatment with CL 316,243, (Bloom et al. 1992) promotes thermogenesis and the appearance of multilocular brown adipocytes in WAT while protecting from high-fat diet induced obesity (Himms-Hagen et al. 1994). Also, infusion of CL 316,243 reduced abdominal fat, increased resting metabolic rate and induced abundant multilocular brown adipocytes expressing UCP1 in WAT of mice (Ghorbani et al. 1997) and Zucker fa/fa rats (Ghorbani and Himms-Hagen 1997). We have recently reviewed the various mechanisms that lead to browning of white fat (Yadav and Rane 2012).

18.2.3 TGF-β Signaling Modulates Brown Adipocyte Induction in WAT

The TGF-β superfamily, inclusive of TGF-β, activins, and BMPs, regulates the developmental programs of many, if not all, diverse cell types (Attisano and Wrana 2002; Feng and Derynck 2005; Massague et al. 2000). TGF-β transmits its signals via dual serine/threonine kinase receptors and transcription factors called Smads, with Smad3 being the principal facilitator of TGF-β isoform derived signals (Feng and Derynck 2005). We recently illustrated an important role of the TGF-β/Smad3 signaling pathway in regulating glucose and energy homeostasis (Yadav et al. 2011). *Smad3*$^{-/-}$ mice exhibit enhanced insulin sensitivity and increased glucose uptake in WAT. When challenged with high-fat diet, *Smad3*$^{-/-}$ mice gained less weight, exhibited enhanced glucose tolerance and insulin sensitivity and were protected from hepatic steatosis. Further, *Smad3*$^{-/-}$ mice displayed significantly reduced fat mass, smaller adipocyte size and reduced expression of WAT-specific genes. *Smad3*$^{-/-}$ mice also exhibited reduced levels of inflammatory cytokines and less inflammatory macrophage infiltration into WAT with a switch in macrophage spectrum from inflammatory M1 to protective M2 macrophages (Lumeng et al. 2007). Interestingly, *Smad3*$^{-/-}$ epididymal WAT possessed a dark-red color and was comprised of smaller multi-locular adipocytes interspersed within larger uni-locular white adipocyte depots (Yadav et al. 2011), a morphology that resembled brown adipocytes (Cousin et al. 1992). Further, we observed that many cells in the *Smad3*$^{-/-}$ WAT stained positive for UCP1 and expressed increased mRNA levels of BAT markers (Seale et al. 2009), i.e. PGC-1α, UCP1, and PRDM16. Physiologically, *Smad3*$^{-/-}$ mice were able to maintain significantly higher body temperature, when exposed to cold for an extended time and displayed elevated rates of fatty acid oxidation.

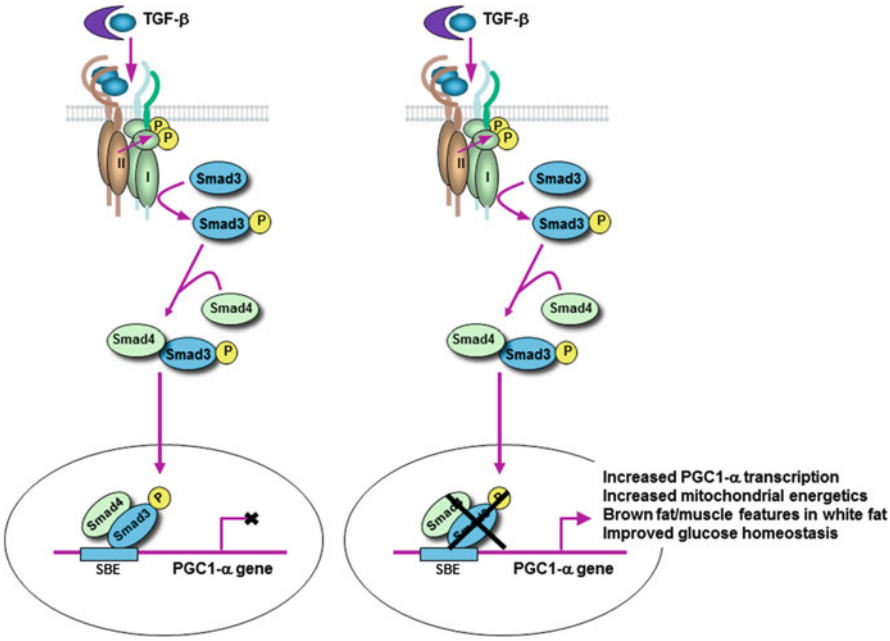

Fig. 18.1 Smad3 regulates PGC1-α promoter in the *white* adipose tissue. Binding of the TGF-β ligand to TβRII results in activation of the TβRI kinase and subsequent phosphorylation of Smad3. Phospho-Smad3 translocates to the nucleus along with Smad4. Smad3 binds to Smad-binding elements (SBE) on the PGC1-α promoter and represses PGC1-α gene transcription (*left panel*). Loss of Smad3 binding to the promoter enhances PGC1-α transcription and is associated with improved functionality of the *white* adipocytes

The molecular signature that we obtained in *Smad3*$^{-/-}$ white fat and in WAT from mice treated with the TGF-β neutralization antibody (1D11) provided interesting insight into the development of brown adipocytes within the WAT. Thus, WAT from *Smad3*$^{-/-}$ mice and mice treated with 1D11 antibodies expressed a preponderance of genes that represent brown fat, mitochondrial and skeletal muscle (Yadav et al. 2011). It is possible that TGF-β acts on a pool of cells that represents a common progenitor for white, brown, and muscle cells. Smad3 co-occupies the genome with cell-type-specific master transcription factors, including Myod1 in myotubes, PU.1 in pro-B cells and Oct4 in ESCs (Mullen et al. 2011). It is probable that in WAT, Smad3 interacts with factor/s that control the induction of cells with BAT/muscle features. Sequence analysis revealed the presence of Smad-binding elements on the promoter of PGC-1α, a transcriptional co-activator that regulates genes involved in energy metabolism and is a direct link between external physiological circuits and the regulation of mitochondrial biogenesis (Lin et al. 2005). Chromatin immunoprecipitation assays provided evidence of Smad3 binding to the PGC-1α promoter (Yadav et al. 2011), and we observed that TGF-β represses the PGC-1α promoter in a Smad3-dependent manner (Fig. 18.1). *Smad3*$^{-/-}$ WAT exhibited increased mitochondrial DNA copy number and functionality. Specifically, the *Smad3*$^{-/-}$ mitochondria

displayed increased rates of basal respiration, which was consistent with the increase in the basal rate of oxygen consumption in intact *Smad3*$^{-/-}$ primary adipocytes. *Smad3*$^{-/-}$ mice also showed increased metabolic rate and exhibited lower respiratory exchange ratio in agreement with the observed increase in mitochondrial function and lipid oxidation.

We also found that elevated TGF-β levels associate with poor metabolic profile in human subjects showing a significant positive association between TGF-β and human adiposity (Yadav et al. 2011). In addition, positive correlations of circulating TGF-β were observed with fat mass, fasting insulin levels, and HOMA insulin resistance index. Importantly, administration of a TGF-β pan neutralization antibody (1D11) suppressed body weight gain, size of fat depots and fat mass, adipocyte cell size and levels of triglyceride, resistin, and leptin in mouse models of obesity and diabetes (Yadav et al. 2011). Further, treatment with 1D11 improved glucose and insulin tolerance, suppressed hyperglycemia and hyperinsulinemia, ameliorated hepatic steatosis, and increased protein levels of BAT/mitochondrial markers in the WAT (Yadav et al. 2011).

The TGF-β superfamily member, BMP7, is implicated in brown adipogenesis (Tseng et al. 2008). BMP proteins promote differentiation to either white adipocytes or brown adipocytes (Schulz et al. 2011). BMP7 triggers commitment of mesenchymal progenitor cells to a brown adipocyte lineage, and implantation of these cells into nude mice results in development of adipose tissue containing mostly brown adipocytes (Tseng et al. 2008). Subpopulation of adipogenic progenitors residing in murine brown fat, white fat, and skeletal muscle were isolated, and it was shown that muscle and white fat derived Sca-1(+) cells were able to differentiate into brown-like adipocytes upon stimulation with BMP7 (Schulz et al. 2011). Also, BMP7 knockout embryos show a marked reduction of brown fat, whereas adenoviral-mediated expression of BMP7 in mice results in a significant increase in brown fat mass and leads to an increase in energy expenditure and a reduction in weight gain (Tseng et al. 2008). However, the role of BMP7 in promoting browning of white fat remains unclear. A more recent study identified Zfp423, a BMP-Smad signaling effector, as a transcriptional regulator of both brown and white preadipocyte differentiation (Gupta et al. 2010), although whether Zfp423 plays a role in promoting brown fat features in white fat is unknown. TGF-β regulates adipocyte differentiation via a Smad3-C/EBP interaction (Choy and Derynck 2003; Choy et al. 2000). Further, Schnurri-2 interacts with Smads and C/EBP during BMP2-regulated adipogenesis (Jin et al. 2006). These studies suggest an important role for TGF-β superfamily members in WAT and BAT biology—one that is analogous to the role played by myostatin during muscle development (McPherron and Lee 2002). Further, the recently described role for TGF-β in glucose-induced cell hypertrophy (Wu and Derynck 2009) may have ramifications in conditions associated with hyperglycemia, glucose intolerance, and insulin resistance.

Circulating TGF-β levels are elevated in cardiovascular disease and hypertension (Gordon and Blobe 2008) and TGF-β and BMI are closely associated in human adipose tissue during morbid obesity (Alessi et al. 2000). Predisposition to certain cancers, atherosclerosis, myocardial infarction, hypertension, and stroke is correlated

with the presence of TGF-β polymorphisms (Grainger et al. 1999). Further, polymorphisms such as the T29C polymorphism, which results in a Leu-Pro substitution at codon 10, are correlated with elevated circulating TGF-β levels. Interestingly, the T29C polymorphism is associated with increased BMI, elevated fasting insulin and glucose levels, and higher HOMA insulin resistance indices (Rosmond et al. 2003). Moreover, the *SMAD3* gene was recently identified in a type 2 diabetes genome-wide association study (Perry et al. 2009) which further supports the notion of TGF-β/Smad3 pathway as a potential target in diabetes and obesity. TGF-β antagonist approaches are being clinically evaluated to treat diseases, such as cancer, fibrosis, scarring, diabetic nephropathy, where elevated TGF-β levels are implicated. The occurrence of elevated TGF-β levels in obese individuals combined with the beneficial effect of the anti-TGF-β neutralization antibody in mouse models of obesity and diabetes offer treatment alternatives for these diseases.

18.2.4 Role of TGF-β Signaling in Pancreatic Islet β-Cell Function

The pancreatic islet β-cell, due to its unique function of insulin synthesis and glucose-stimulated insulin secretion (GSIS), is a prime target of affliction in diabetes (Weir and Bonner-Weir 2004). In addition, a majority of patients with diabetes mellitus type II develop β-cell dysfunction in concert with insulin resistance in target organs of insulin action: liver, muscle, and adipose tissue (Herman and Kahn 2006). Improved mechanistic understanding of normal β-cell function and insulin action is needed to enable early diagnosis of β-cell dysfunction and insulin resistance and to facilitate development of new rational therapeutics for diabetes.

Development of the endocrine and exocrine pancreas is controlled by factors that include members of the TGF-β superfamily (Kim and Hebrok 2001; Kim and MacDonald 2002). In addition, TGF-β signaling has been implicated in pancreatic diseases (Rane et al. 2006). BMP signaling plays an instructive role during early pancreatic development (Kim and Hebrok 2001; Kim and MacDonald 2002; Rane et al. 2006) and regulates mature β-cell function (Gannon 2007; Goulley et al. 2007), whereas activin signaling regulates islet morphogenesis and β-cell mass (Yamaoka et al. 1998; Zhang et al. 2004). TGF-β isoforms are expressed in the epithelium and mesenchyme of embryonic pancreas and in adult pancreas (Yamanaka et al. 1993). Islet cells demonstrate diffuse cytoplasmic immunostaining for TGF-β isoforms with most of the positive islet cells co-expressing insulin. TGF-β receptors (TβRI and TβRII) are present in the pancreatic epithelium and mesenchyme during early stages of development and postnatally in pancreatic islets and ducts. Further, Smad proteins are expressed in the pancreas which elucidates that components needed for activation of the canonical TGF-β signaling exist within the pancreas.

Disruption of TGF-β signaling at the receptor level using mice expressing the dominant-negative TGF-β type II receptor (DNTβRII) resulted in increased

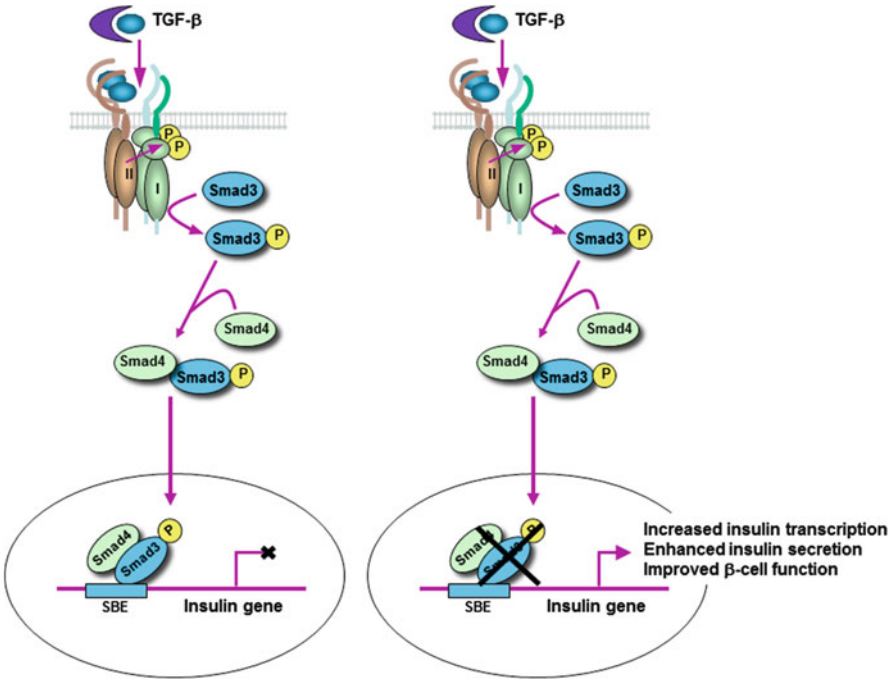

Fig. 18.2 Smad3 regulates the insulin promoter in pancreatic islet β-cells. Binding of the TGF-β ligand to TβRII results in activation of the TβRI kinase and subsequent phosphorylation of Smad3. Phospho-Smad3 translocates to the nucleus along with Smad4. Smad3 binds to Smad-binding elements (SBE) on the Insulin promoter and represses Insulin gene transcription (*left panel*). Loss of Smad3 binding to the promoter enhances Insulin transcription and is associated with improved functionality of the pancreatic islet β-cells

proliferation of pancreatic acinar cells and severely perturbed acinar differentiation (Bottinger et al. 1997). Additionally, DNTβRII mice exhibit increased endocrine precursors and proliferating endocrine cells, with an abnormal accumulation of endocrine cells around the developing ducts of mid-late stage embryonic pancreas (Tulachan et al. 2007). Transgenic mice expressing TGF-β in β-cells exhibit abnormal small islet cell clusters without formation of normal adult islets, although the overall islet cell mass is not significantly diminished (Lee et al. 1995).

We recently described an important role of the TGF-β/Smad3 pathway in regulation of insulin gene transcription and β-cell function (Lin et al. 2009). We identified insulin as a TGF-β target gene and showed that Smad3 occupies the insulin gene promoter and represses insulin gene transcription (Fig. 18.2). In contrast, Smad3 siRNAs relieve insulin transcriptional repression and enhance insulin levels. Transduction of adenoviral Smad3 into primary human and nonhuman primate islets suppresses insulin content, whereas dominant-negative Smad3 adenoviral transduction enhances insulin levels. Consistent with this, Smad3-deficient mice exhibit moderate hyperinsulinemia and mild hypoglycemia. Moreover, Smad3

deficiency results in improved glucose tolerance and enhanced GSIS in vivo. In ex vivo perfusion assays, Smad3-deficient islets exhibited improved glucose-stimulated insulin release. Interestingly, Smad3-deficient islets harbor an activated insulin-receptor signaling pathway and TGF-β signaling regulates expression of genes involved in β-cell function. These studies emphasize TGF-β/Smad3 signaling as an important regulator of insulin gene transcription and β-cell function and suggest that components of the TGF-β signaling pathway may be dysregulated in diabetes.

We find that inhibition of Smad3 signaling leads to elevated GSIS ex vivo and in vivo. It is plausible that the effects of TGF-β/Smad3 on insulin gene transcription may be independent of its role in GSIS. Levels of expression of genes involved in insulin biosynthesis, pro-insulin processing, glucose sensing, glucose metabolism, incretin signaling, insulin exocytosis, and GSIS are significantly elevated in *Smad3*$^{-/-}$ islets and, conversely, significantly repressed in cells harboring a constitutively active TβRI/Smad3 pathway. Interestingly, we observe an activated insulin receptor signaling pathway in *Smad3*$^{-/-}$ islets, which is consistent with the enhanced β-cell function in *Smad3*$^{-/-}$ mice.

TGF-β appears to exert a bimodal effect on GSIS depending on the dose, time of exposure, and concentration of coexisting glucose (Sekine et al. 1994; Sjoholm and Hellerstrom 1991). Therefore, at low glucose concentration TGF-β stimulates insulin release, whereas at a high glucose concentration the same dose of TGF-β significantly inhibits GSIS (Sjoholm and Hellerstrom 1991; Sekine et al. 1994). Interestingly, dual and opposing roles of TGF-β in cancer are believed to suppress primary tumors and paradoxically promote cancer metastases (Roberts and Wakefield 2003). Recent reports of the role of BMP4/BMPR1A and Smad7 in β-cell function further underscore the important role of TGF-β superfamily signaling in this cell type (Smart et al. 2006; Bertolino et al. 2008; Goulley et al. 2007). BMP4-BMPR1A signaling plays a key role in insulin secretion by positively regulating genes involved in glucose sensing, glucose-metabolism-coupled secretion, incretin signaling, proinsulin processing, and insulin exocytosis (Goulley et al. 2007). Our findings of TGF-β/Smad3 mediated regulation of genes associated with β-cell function are consistent with an important role of the TGF-β superfamily signaling in pancreas development, particularly, β-cell function.

The findings that amplified TGF-β/Smad3 signals repress insulin transcription and reduce insulin level and insulin secretion, whereas ablation of Smad3 leads to improved β-cell function suggesting that abnormal TGF-β signaling may promote pathogenesis of dysfunctional β-cells. We propose that TGF-β/Smad3 pathways may (Sekine et al. 1994) regulate β-cell function in settings of increased insulin demand, including insulin resistance, obesity, and during β-cell injury. Further, our results that reduced TGF-β/Smad3 signaling markedly enhance insulin content and insulin secretion suggest that pharmacological inactivation of TGF-β/Smad3 signaling might be useful for promoting β-cell differentiation and ameliorating β-cell failure during diabetes.

18.3 Perspectives and Conclusion

Together, our findings support the notion that the TGF-β signaling network plays important roles in the functionality of the pancreatic β-cell and that of the adipose tissue (Figs. 18.1 and 18.2). Further, we propose that this pathway serves an integral role in metabolic homeostasis. We believe that this role is not limited to the TGF-β isoforms and that there is evidence supporting an integrated role for various TGF-β superfamily isoforms in the process. In addition, we believe that other signaling pathways that intersect into, or, are off-shoots of, the TGF-β signaling network play a collaborative role to maintain metabolic homeostasis. As such, it is important to define the precise role of TGF-β signaling in regulating the actions of various metabolic organs—both via the canonical Smad-transcription factor loop and/or via non-canonical signaling pathways. TGF-β levels are elevated in metabolic disease, which supports the utility of therapeutics aimed at targeting the TGF-β pathway for these diseases. However, the complexity of the TGF-β signaling network warrants a cautious and rational approach to designing anti- TGF-β modalities to combat metabolic diseases.

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Part V
Disease Prognosis and Therapy

Chapter 19
Targeting Pro-Angiogenic TGF-β Signaling in the Tumor Microenvironment

Kristian Pietras and Sara I. Cunha

Abstract The recently developed targeted anti-angiogenic agents have been introduced into clinical practice over the course of the past decade. High hopes were placed on targeting the VEGF signaling pathway in endothelial cells following the preceding successful drug development in the preclinical setting. Indeed, the therapeutic efficacy observed in mouse models of cancer has in some cases been translated into clinical benefit for patients. Nevertheless, many anti-angiogenic therapies have failed to provide substantial improvement in survival in large phase III clinical trials. In the search for attractive and complementary angiogenic signaling pathways, the TGF-β family stands out as one of the most interesting. Our expanding knowledge on TGF-β signaling in the tumor vasculature has led to the development of specific inhibitors targeting TGF-β, ALK1, and endoglin. Many clinical trials exploring the concept of targeting pro-angiogenic TGF-β signaling are currently underway, and preliminary reports are encouraging. Here, we will discuss opportunities and challenges of targeting the TGF-β system for anti-angiogenic therapy of cancer.

Keywords Angiogenesis • BMP • Cancer • Targeted therapy • TGF-β

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Abbreviations

ALK	Activin receptor-like kinase
BMP	Bone morphogenetic proteins
EC	Endothelial cell
HHT	Hereditary hemorrhagic telangiectasia
TGF-β	Transforming growth factor-β
VEGF	Vascular endothelial growth factor

19.1 Introduction

Based on several successful preclinical studies, the oncologic clinical panorama anticipated with hope the use of anti-angiogenic therapies to combat cancer. Nevertheless, the introduction of vasculature targeting drugs led to limited clinical benefit measured in months, with almost negligible effect on overall survival and mainly in combination with cytotoxic agents. Recent studies provide evidence for the development of resistance and acquisition of a more aggressive and invasive phenotype in tumors that have been treated with vascular endothelial growth factor (VEGF) anti-angiogenic approaches. This points to the need for additional examination of the biology of tumor-nurturing blood vessels, in order to accurately pinpoint promising and novel anti-angiogenic targets. Furthermore, it is reasonable to assume that curing cancer is a complex task that requires combined strategies aiming at different tumor cellular compartments, in addition to aiming at the ample spectrum of signaling networks that are commonly deregulated in malignant cells.

Often altered in neoplasms and involved in multiple cellular functions, the large transforming growth factor-β (TGF-β) family is, as a result, an obvious candidate for aiming such efforts. More specifically, because TGF-β signaling encompasses a number of vascular restricted receptors, including activin receptor-like kinase (ALK)1 and endoglin, they represent attractive anti-angiogenic therapeutic targets for cancer. For a thorough summary of endothelial cell signaling by components of the TGF-β family, please see Chap. 14.

19.2 TGF-β Signaling as Tumor Angiogenesis Targets

19.2.1 Clinical Relevance of ALK1 in Cancer

ALK1 expression in the early developing mouse embryo coincides with sites of vasculo- and angiogenesis (Roelen et al. 1997), with prevailing expression in developing arterial endothelium, while nearly absent from small capillaries (Seki et al. 2006). During early mouse development ALK1 is strongly expressed, whilst it tends

to be concealed in the adult quiescent vasculature. The expression of ALK1 is reversibly turned on during neo-angiogenesis events in wound healing or in tumors (Seki et al. 2006).

Information about pattern and intensity of ALK1 expression in human normal and cancerous tissues is unfortunately still very scarce. The public Human Protein Atlas program (Ponten et al. 2008) has characterized ALK1 immunostaining of both normal and neoplastic tissues. In this study, ALK1 exhibited frequent strong expression most notably in neuronal cells of the cerebral cortex, hippocampus, ventricle, and cerebellum, in the gall bladder, GI tract, and in tubular cells in the kidney, in line with the murine ALK1 expression profile (Panchenko et al. 1996; Wu et al. 1995). The same organization identified ALK1 prevailing expression in neoplasms in the colorectal tract, pancreas, stomach, and thyroid, as well in malignant lymphomas and melanomas. A preliminary descriptive study reported a weak but widespread pattern of expression in the vasculature of normal tissues, including positive staining in lymphatic tissues, lung, intestines, and pancreas. In a follow-up study by the same entity, ALK1 was found to be extensively present on tumor blood vessels, especially in lymphomas, and malignant tissues of the prostate, skin, thyroid, kidney, ovary, lung, pancreas, and liver (Hu-Lowe et al. 2011). Thorough studies analyzing the prognostic strength and diagnostic significance of ALK1 are highly warranted.

19.2.2 Inhibitory Drugs Targeting ALK1

Given the extensive literature describing paradoxical effects of signaling stemming from ALK1 in endothelial cells (ECs), the prediction for the net outcome for acute inhibition of ALK1 in the context of cancer is an intricate task. Furthermore, the complex ligand-receptor binding specificity and/or redundancy within the TGF-β family add multiple hurdles to the estimate of therapeutic efficacy, benefit and secondary effects for the various ALK1 inhibitors currently under development.

Small molecules comprising broad inhibitory spectrum against bone morphogenetic protein (BMP) type I receptor kinases, including ALK1, have been generated. Smad-dependent and -independent signaling, induced by BMP can be blocked by compounds, such as dorsomorphin and its structural derivative LDN-193189 (Boergermann et al. 2010; Yu et al. 2008b). These drugs can be useful and potent in inhibiting BMP type I receptor signaling in a range of diseases and familial syndromes (Yu et al. 2008a); however, their effect on BMP-induced tumor angiogenesis remains to be determined. Nonselective inhibitors also raise the concern of off-target effects, as documented for dorsomorphin (Cannon et al. 2010; Vogt et al. 2011). Further development of small molecule compounds with a more specific inhibition profile aiming primarily at BMP type I receptors, and specifically towards ALK1, should be considered.

Antibodies or soluble extracellular receptor domain traps use may alternatively provide a tighter inhibition of specific ALK1 activity. ALK1 inhibitors are currently

under development for cancer treatment as anti-angiogenic drugs. Up to now, several biological inhibitors against ALK1 have been generated for in vivo use. Firstly, Pfizer is currently conducting phase I-trials of PF-3446962, a fully human monoclonal antibody against ALK1. Secondly, the use of an ALK1-Fc fusion protein (amino acids 23–119 of mouse ALK1) was reported by Genentech studies on hematogenous and lymphatic vessel development in the mouse (Niessen et al. 2010). Another ALK1 targeting agent is ACE-041 (mouse counterpart RAP-041, amino acids 22–117 of mouse ALK1), a human ALK1-Fc fusion protein, currently undergoing a phase II clinical trial, coordinated by Acceleron Pharma.

19.2.3 PF-3446692

Preclinical tumor studies using PF-3446962 were recently reported (Hu-Lowe et al. 2011). ALK1 blockade exhibited attenuation of VEGF-induced EC proliferation and tube formation in vitro. In addition, therapeutic treatment with the ALK1-neutralizing antibody delays tumor growth in vivo in MDA-MB-231 breast carcinoma and M24met/R melanoma mouse models.

The anti-hALK1 antibody from Pfizer has completed phase I clinical trials for patients with advanced solid tumors. Preliminary evidence from the trial indicates that the anti-hALK1 antibody reduced the amount of ALK1-positive circulating ECs, which were found to be present in increased levels in advanced stage cancer patients. The PF-3446962 antibody was well tolerated up to 10 mg/kg lacking concerning adverse events in all 44 patients participating in the phase I trial. The most common side effects included transient thrombocytopenia and asymptomatic elevation of pancreatic enzymes. Hence, preliminary observations evoke encouraging clinical activity where three partial responses were observed in patients who have previously received fruitless anti-angiogenic regimens.

The mechanism of action of this antibody has recently been described (van Meeteren et al. 2012). The anti-hALK1 antibody selectively recognizes human ALK1 and interferes with BMP9-induced signaling in ECs. Moreover, the anti-hALK1 antibody competitively obstructs BMP9 and TGF-β binding to ALK1 receptor and prevents BMP9-dependent recruitment of endoglin into the angiogenesis-mediating signaling complex, which may eventually hinder the BMP9/ALK1 proangiogenic effects.

19.2.4 ACE-041/RAP-041/dalantercept

The effects of RAP-041 were recently analyzed in the RIP1-Tag2 transgenic mouse model of pancreatic neuroendocrine tumorigenesis (Cunha et al. 2010). The RIP1-Tag2 tumors readily express ALK1 exclusively on ECs, mirroring the expression profile of common vascular markers during tumor progression.

RIP1-Tag2 mice treated with 2 weekly doses between 1 and 12 mg/kg RAP-041 results in a dose-dependent retardation of tumor growth and the highest dose effectively prevents further tumor expansion. Incidentally, Alk1 heterozygous mice under the RIP1-Tag2 context recapitulated the effects obtained by systemic inhibition by RAP-041. Specificity of the ALK1-targeting drug treatment was validated by decreased expression of ALK1 downstream target genes in tumors from mice treated with the RAP-041. Alternative studies with the same drug demonstrated that RAP-041 possesses growth-inhibitory response in orthotopic MCF-7 breast carcinomas (Mitchell et al. 2010).

Acceleron Pharma recently concluded a phase I trial for the human ALK1-Fc fusion protein ACE-041, which interestingly only binds and neutralizes BMP9 and BMP10, but not to any of the TGF-β isoforms (Cunha et al. 2010; Mitchell et al. 2010). Thirty seven patients with solid tumors or refractory multiple myeloma were recruited to assess safety and tolerability of ACE-041, as well as changes in tumor metabolism evaluated by ^{18}F deoxyglucose positron emission tomography. ACE-041 regimen included an administration every three weeks and was well tolerated at doses up to 1.6 mg/kg. Common collateral effects comprised peripheral edema, fatigue, nausea, headache, anorexia, and anemia. However, toxicities usually associated with VEGF inhibition, such as hypertension, proteinuria, GI tract perforation, and hemorrhaging, were not observed, possibly because ALK1 is predominately present in the actively cycling endothelium within the tumor milieu and preferentially localizes in arterial endothelium, whereas VEGFRs present a more global distribution (Seki et al. 2003).

In this clinical study, one patient with refractory head and neck squamous carcinoma exhibited a partial response (>30 weeks) and six other patients exhibited stable disease. Rapid reduction in tumor metabolic activity (>20%) was observed in ten patients, measured by FDG-PET scanning. Of note, many of the patients included in this trial had been previously inefficiently treated with other therapeutic regimens, including VEGF-targeting drugs.

After such an encouraging phase I clinical trial, the ALK1 inhibiting agent ACE-041 is undertaking a phase II clinical study with an expanded cohort on head and neck squamous carcinoma patients (http://clinicaltrials.gov). Apart from its anticancer specification, ACE-041, is further being developed for testing in age-related macular disease treatment.

19.2.5 Possible Side Effects from ALK1 Inhibition

Anti-angiogenic therapies, mainly in the form of inhibitors of VEGF signaling, have been in routine clinical use for several years for various malignancies. Side effects from inhibiting angiogenesis are in general milder than those arising from conventional chemotherapeutic treatment and include bleeding, hypertension, fatigue, and nausea. Specifically, given the causal relationship between impaired ALK1 signaling and hereditary hemorrhagic telangiectasia (HHT)-related symptoms,

inhibition of ALK1 signaling in the vasculature may induce de novo arteriovenous malformations and hemorrhaging. In fact skin telangiectasis was observed in a patient treated with ACE-041, validating the on-target effect of the drug and indicating that the appearance of telangiectases may be useful as a surrogate marker for efficacy. Loss-of-function mutations in ALK1 linked with hereditary pulmonary arterial hypertension (Machado et al. 2009) highlight the risk for pulmonary circulation hemodynamics perturbations with ALK1 inhibitors. Interestingly, complete blockade of ALK1 signaling triggered by both BMP9 and BMP10 resulted in lung hemorrhaging (Ricard et al. 2012), an organ that should thus be primarily monitored. Furthermore, given the high expression levels of BMP9 in the liver, this organ should be also carefully monitored (Bidart et al. 2012). Finally, as ALK1 is reported to be expressed by, and conceivably important for lymphatic ECs, cells of the pituitary gland, hepatic stellate cells, chondrocytes, and pancreatic ductal cells, special care should be taken to record adverse events from the treatment of ALK1 inhibitors related to processes regulated by these particular tissues (Alexander et al. 1996; Finnson et al. 2008; Niessen et al. 2010; Ungefroren et al. 2007; Wiercinska et al. 2006).

19.3 Clinical Relevance of Endoglin in Cancer

Endoglin positive intratumoral microvascular density strongly correlates with poor prognosis in cancer, being associated with shorter survival and relapse-free survival rates (Bernabeu et al. 2009; Fonsatti et al. 2010). Moreover, subcutaneous tumor neovascularization and growth are impaired in endoglin heterozygous mice, reiterating the relevance of endoglin in tumor angiogenesis (Duwel et al. 2007). An enormous body of literature highlights the potential of endoglin as a tumor vasculature marker in preclinical and clinical studies (Bredow et al. 2000; Costello et al. 2004; Fonsatti et al. 2000; Fonsatti et al. 2010). In this respect, endoglin can be a more specific marker for new, immature tumor vessels, unlike other EC markers, which are expressed in both mature and immature vessels (Beresford et al. 2006).

High levels of endoglin expression have therefore been confirmed in several experimental models, such as breast, prostate, and colorectal cancer, for example (Akagi et al. 2002; Beresford et al. 2006; Wikstrom et al. 2002).

Endoglin expression has also been associated with predisposition of colorectal mucosa dysplastic tissues evolution into fully developed carcinomas (Akagi et al. 2002). Furthermore, in prostate cancer endoglin-positive microvessel density correlated with Gleason score, metastasis and tumor stage (Wikstrom et al. 2002). Paradoxically, the same study indicated that endoglin-positive vessels were more poorly covered by α-smooth muscle cells and correlated with survival. Another study in colorectal cancer patients reported that vessel density evaluated by endoglin staining significantly correlated with survival. Additionally, other reports correlate loss of endoglin with prostate cancer progression and aggressiveness

(Liu et al. 2002) and endoglin presence with decrease in prostate tumor cell motility (Craft et al. 2007). Our own work strengthens the vascular role of endoglin as protective against tumor cell metastatic seeding (Anderberg et al. 2013).

Endoglin exists in the body in two different forms: membrane-bound and circulating (Bernabeu et al. 2009). Levels of soluble endoglin have been reported in plasma of pregnant women suffering from preeclampsia (Venkatesha et al. 2006) and also in patients suffering from colorectal, breast, prostate, and leukemic cancers, correlating positively with metastatic disease (Fujita et al. 2009; Karam et al. 2008; Li et al. 2000; Takahashi et al. 2001b). However, the role of soluble endoglin in cancer is still poorly understood. Since soluble endoglin contains the binding site for different ligands of the TGF-β family, it may act as a scavenger of circulating ligands, preventing their binding to the functional receptors, hence interfering with vascular function and angiogenesis (Perez-Gomez et al. 2010). An intriguing question concerns the source of soluble endoglin detected in cancer patients. Since endoglin levels are higher in tumor vessels, soluble endoglin may conceivably derive from shedding from tumor ECs and, importantly, it may represent a surrogate marker of angiogenic activity (Fonsatti et al. 2003).

19.3.1 TRC105

The potential of endoglin-targeting monoclonal antibodies to be used as a therapeutic anti-angiogenic strategy in human cancer has received considerable support from preclinical studies.

Intravenous systemic administration of anti-endoglin monoclonal antibody TRC105 was shown to suppress angiogenesis, tumor growth, and metastasis without overt toxicity in mice (Seon et al. 1997; Tabata et al. 1999; Takahashi et al. 2001a). The combination of endoglin-targeting antibody with cyclophosphamide and doxorubicin was reported to exhibit synergistic antitumor efficacy in human skin/SCID mouse chimeras, including in metronomic regimens (Shiozaki et al. 2006; Takahashi et al. 2001a).

A total of 50 patients with advanced refractory cancer disease were included in TRC105 phase I clinical trial (Seon et al. 2010). Doses up to 1 mg/kg were administered every other week to assess efficacy, toxicity, and tolerability of TRC105. One patient with castrate-refractory prostate cancer remained in the study even after 3 years of TRC105 at 0.01 mg/kg with a complete response and bone scan normalization. In addition, an ovarian cancer patient presented with 6-month stable disease. Dose-limiting toxicities incorporate GI hemorrhage and anemia.

A phase I clinical trial has been initiated with breast metastatic cancer patients to determine maximum tolerated dose of TRC105 in combination with capecitabine, a DNA synthesis blocking agent, approved by FDA as adjuvant treatment for colon cancer, as well as, for metastatic breast and colorectal cancers (www.clinicaltrials.gov).

19.3.2 Endoglin-Fc

More recently, Acceleron Pharma characterized a soluble mouse and human endoglin extracellular domain fused to an immunoglobulin Fc domain (human endoglin amino acid sequence 26–359). This endoglin ligand trap binds specifically and with high affinity to BMP9 and BMP10 in vitro. This agent significantly impaired VEGF-induced chick chorioallantoic membrane assay in vivo. Finally, murine soluble endoglin extracellular domain acted as an anti-angiogenic factor decreasing blood vessel sprouting in VEGF/fibroblast growth factor-induced angiogenesis in in vivo angioreactors and tumor burden in the colon-26 mouse tumor model (Castonguay et al. 2011). Together these findings indicate an important role for soluble endoglin in the regulation of angiogenesis and evoke the prospective efficacy of endoglin-Fc as an anti-angiogenic therapeutic agent.

19.3.3 Possible Side Effects of Targeting Endoglin

Only two dose-limiting toxicities were reported in patients who received TRC105: one grade 4 gastric ulcer bleeding in a patient treated with 0.1 mg/kg after 4 days, which resolved spontaneously and one patient experienced grade 3 infusion reaction.

Based on a recent study, a careful follow-up of the patients treated with endoglin targeting antibodies should be in place, since the preclinical studies in mouse models, either endoglin heterozygous or endoglin-EC specifically ablated led to the emergence of worsened phenotypes with tumors acquiring a refractory behavior with increased metastatic seeding (Anderberg et al. 2013).

19.4 Clinical Relevance of TGF-β Signaling in Cancer

The role of TGF-β in cancer biology is complex and controversial, involving aspects of tumor suppression as well as tumor promotion. The ability of TGF-β to potently inhibit the proliferation of epithelial, endothelial, and hematopoietic cell lineages is central to the tumor-suppressive mechanism (Yingling et al. 2004). For example, the TGF-β receptors and their Smad signaling mediators are tumor suppressors that frequently become inactive in gastrointestinal, pancreatic, ovarian, and hepatocellular carcinomas, as well as in subsets of gliomas and lung adenocarcinomas (Bierie and Moses 2006a). As tumors evolve, they often become noncompliant to TGF-β-mediated growth inhibition and overexpress TGF-β, which in turn has a manifested autocrine impact on the biology of the malignant cells themselves. A tumor microenvironment that favors tumor growth and metastasis in a paracrine fashion is

therefore created. Interestingly, in breast carcinoma, glioblastoma, melanoma, and other cancer types, specific loss of TGF-β-mediated growth inhibitory responses often accumulate through alterations downstream of Smad, leaving the rest of the TGF-β pathway operational and available to co-opt in detriment of tumor progression (Massague and Gomis 2006).

19.4.1 Inhibitory Drugs Targeting TGF-β

Improvements in understanding how TGF-β impinges upon the tumor microenvironment have led to the development of TGF-β inhibitors for cancer treatment. Three categories of TGF-β inhibitors have been characterized: soluble antisense oligonucleotides, monoclonal antibodies, and small molecule inhibitors. At first sight, this pathway presents an attractive target for the development of cancer therapeutics that simultaneously attacks the tumor and its microenvironment. One could, thus, anticipate that TGF-β targeting drugs would have a very efficacious effect on malignant progression. Nonetheless, the ubiquitous and pleiotropic nature of TGF-β signaling and its dual role in tissue homeostasis and in tumorigenesis pose on this kind of inhibitory strategies a risk that cannot be underestimated in preclinical and clinical drug development programs. Multiple clinical trials targeting the TGF-β pathway are currently in progress (see Table 19.1).

19.4.2 TGF-β Antisense Compounds

The specificity of hybridization draws antisense oligonucleotides as targeted and functional therapeutic tools to selectively modulate the expression of a variety of genes involved in the pathogenesis of malignancies and other genetic diseases (Stahel and Zangemeister-Wittke 2003; Tamm et al. 2001; Tamm and Wagner 2006).

Trabedersen (AP-12009), developed by Antisense Pharma GmbH, is a synthetic antisense oligodeoxynucleotide designed to block the production and tumorigenic effects of TGF-β2. Trabedersen is indicated for the treatment of malignant brain tumors and other TGF-β2 overexpressing solid tumors, such as those of the skin, pancreas, and colon. Preclinical studies provided evidence that trabedersen reduced the secretion of TGF-β2 in cultured tumor cells and exhibited antitumor activity ex vivo. Chronic intracerebral or systemic administration of trabedersen does not cause life-threatening collateral effects in animals, confirmed in early clinical trials in advanced cancer patients. In the initial phase I and II open-label dose-escalation study the compound was able to significantly prolong the median time to relapse compared with the published relapse times for temozolomide chemotherapy (Bierie and Moses 2006b).

Table 19.1 Summary of TGF-β family targeting clinical trials implicated in cancer

Drug target	Malignant condition	Intervention	Study type	Status	Evaluations
TGF-β2	Advanced metastatic carcinoma	Trabedersen with GM-CSF gene modified autologous tumor cell vaccine	Phase I	Completed	Primary outcome: – Safety Secondary outcomes: – Time to progression – Effect on immune stimulation
TGF-β2	Pancreatic neoplasms Melanoma Colorectal neoplasms	Trabebersen	Phase I	Completed	Primary outcome: – Maximum tolerated dose – Dose-limiting toxicity Secondary outcomes: – Safety and tolerability – Plasma pharmacokinetics – Method to assess the urine Pharmacokinetic profile – Effect on TGF-β2 plasma levels – Antitumor activity:effect on tumor size and tumor markers
TGF-β2	Glioblastoma Anaplastic astrocytoma	Trabedersen	Phase IIb	Completed	Primary outcome: – Overall response rate evaluated by brain MRI scans Secondary outcomes: – Overall survival – Response rates – Progression-free survival – Time to progression – Time to response – Survival and variation of tumor size on brain MRI – Change in quality of life – Safety and tolerability

TGF-β2	Anaplastic astrocytoma Glioblastoma	Trabedersen	Phase III	Completed	Primary outcome: – Survival rate Secondary outcomes: – Progression rate – Time to death – Overall response rate – Tumor control rate – Duration of response – Time to progression – Survival rate – Quality of life
TGF-β	Advanced malignant melanoma or renal cell carcinoma	GC1008	Phase I	Completed	Primary outcome: – Maximum tolerated dose – Dose-limiting toxicity – Safety Secondary outcomes: – Pharmacokinetic and dynamic
shFurin	Colon carcinoma with liver metastasis	FANG vaccine	Phase II	Recruiting	Primary outcome: – Overall survival Secondary outcome: – Immune response
shFurin	Advanced melanoma	FANG vaccine	Phase II	Recruiting	Primary outcome: – Immune response Secondary outcome: – Survival

(continued)

Table 19.1 (continued)

Drug target	Malignant condition	Intervention	Study type	Status	Evaluations
TGF-β1, 2, 3	Metastatic breast cancer	GC1008 + Radiation	Safety	Recruiting	Primary outcome: – Adverse events Secondary outcome: – Local response rate by PET/CT
TGF-β RI	Malignant glioma	LY2157299 with Temozolomide-based Radiochemotherapy	Phase Ib/2a	Recruiting	Primary outcome: Phase 1b – Safety and tolerability – dose Primary outcome: Phase 2a – Confirm tolerability – Pharmacodynamics
TGF-β 1, 2, 3	Relapsed malignant pleural mesothelioma	GC1008	Phase II	Ongoing	Primary outcome: – 3-month PFS rate Secondary outcomes: – Progression-free survival – Immune response parameters

ALK1	Advanced solid tumors	PF-03446962	Phase I	Recruiting	Primary outcomes: – Maximum tolerated dose Secondary outcomes: – Pharmacokinetics – Evaluation of immunogenicity – Evidence of antitumor activity – Effect on vascular function – Circulating ECs, circulating endothelial progenitors, and soluble proteins related to ALK1 signaling – Safety
ALK1	Squamous cell carcinoma of the head and neck, renal cell carcinoma, endometrial cancer Glioblastoma, renal cell carcinoma, breast carcinoma, others	ACE-041/dalantercept	Phase II	Recruiting	Primary outcome: – Response rate Secondary outcome: – Adverse events – Serum concentration – Progression-free survival – Overall survival – Time progression – Duration of response – Disease control rate – Expression of ALK1 in tumor tissue and blood
Endoglin		TRC105	Phase I/II	Recruiting	Primary outcome: – Progression-free survival Secondary outcome: – Response rate

In a phase IIb trial, improved survival was observed in refractory high-grade gliomas patients who were administered trabedersen intratumorally, by convection-enhanced delivery (Bierie and Moses 2006b; Vallieres 2009; Yingling et al. 2004). One hundred and forty-five patients with histopathology of recurrent/refractory glioblastoma multiforme or anaplastic astrocytoma were randomly assigned to receive trabedersen or standard chemotherapy (Bogdahn et al. 2011). One patient achieved a complete response in all tumor sites after a single cycle of trabedersen, and several patients achieved tumor reductions of more than 80 % (Bogdahn et al. 2011; Yingling et al. 2004). In addition, disease stabilized in seven out of 24 patients, and two patients were in complete remission after treatment. However, this observation requires validation by an ongoing large-scale phase III randomized controlled trial (Vallieres 2009). Meanwhile, continued research on trabedersen should help determine the roles of TGF-β2 in cancer. As a result of these studies, trabedersen received orphan drug status in the European Union in 2002.

Overexpression of TGF-β2 in pancreatic malignancies is suggested to be a pivotal factor for malignant progression by inducing proliferation, immunosuppression, angiogenesis, and metastasis. Antitumor activity was reported for trabedersen in human pancreatic cancer cells and in an orthotopic xenograft mouse model of metastatic pancreatic cancer (Schlingensiepen et al. 2010), significantly reducing tumor growth, lymph node metastasis, and angiogenesis. According to these promising results, trabedersen appears attractive for the treatment of pancreatic adenocarcinoma but warrants further clinical insight.

19.4.3 TGF-β Antibodies

GC1008, the only TGF-β antibody in clinical trials, is a human IgG4 monoclonal antibody capable of neutralizing all TGF-β isoforms. Cohorts of patients with advanced malignant melanoma or renal cell carcinoma, who had failed at least one prior therapy were treated to assess effectiveness with GC1008 at doses from 0.1 to 15 mg/kg. Twenty-two patients were included (21 malignant melanoma, one renal cell carcinoma) and treated in this trial. No dose-limiting toxicities were observed and the highest dose of GC1008 was determined to be safe. Adverse events included skin rash, fatigue, headache, epistaxis, gingival bleeding, and gastrointestinal symptoms. So far, five patients reached stable disease and received extended treatment. Out of these five responders, three patients presented with metastasis shrinkage in the liver and other sites. One melanoma patient achieved a partial response with a lesion reduction of >75 %. All in all, GC1008 is well tolerated and neutralization of all TGF-β isoforms seems to hold promise in the treatment of one of the most aggressive cancer types, for which a phase II protocol is being expanded.

19.4.4 Small-Molecule Kinase Inhibitors Targeting TGF-β Receptors

There are several well-characterized small-molecule kinase inhibitors identified to target the TGF-β pathway, including A-80-01, LY364947, LY580276, LY566578, SB-505124, SD-093, SD-208, and SB-431542 (Bierie and Moses 2006a). The above ALK5 small-molecule inhibitors can, in general, also target ALK4 and ALK7 receptor, so results obtained through their application might not directly correlate with TGF-β/ALK5-specific signaling and dissecting specific effects stemming from each of the three receptors may require substantial validation (DaCosta Byfield et al. 2004; Inman et al. 2002; Peng et al. 2005). The inhibition effect on ALK4 and ALK7, in addition to the TGF-β-specific ALK5 effect on tumorigenesis, is yet to be determined. In particular ALK4 could be important, as its upregulation in the MMTV-Neu mouse model has been shown to correlate with activated Smad2 and loss of ALK5 expression (Landis et al. 2005). Furthermore, in the absence of ALK5, ALK4 mediates Smad2 phosphorylation and consequently αSMA expression during mouse yolk sac vasculogenesis (Carvalho et al. 2007).

Many of the TGF-β inhibitors were tested on breast and lung cancer xenograft tumor-bearing mice demonstrating significant tumor growth delay, providing supporting evidence for further development of these kinase inhibitors for clinical investigation (Lahn et al. 2005).

Treatment of syngeneic R3T or 4T1 tumor-bearing mice with orally supplied SD-208 inhibited primary tumor growth, angiogenesis, and number and size of metastasis. In contrast, SD-208 failed to inhibit R3T tumor growth or metastasis in athymic nude mice, suggesting that the antitumor effect is predominantly affecting immune responses (Ge et al. 2006).

SD-208 also inhibited kinase activity in SMA-560 gliomas in syngeneic mice, exhibiting a 25 % survival advantage in treated animals. No effect was detected on microvascular density. However, histological analysis of SD-208 treated tumors revealed an increase of infiltrated NK cells, CD8 T cell, and CD11b positive macrophages and neutrophils into responding tumors despite the negligible effects on overall tumor burden (Uhl et al. 2004).

Consistent with these observations, TGF-β signaling inhibition with SD-208 in the Panc-1 orthotopic pancreatic cancer model showed significant reduction of primary tumor weight and decreased incidence of metastasis. Histological evaluation revealed that SD-208 treatment reduced proliferation and induced apoptosis and vessel density in the tumor microenvironment. Additionally, an immune system contribution was observed with a greater B-cell infiltration in SD-208-treated tumors (Gaspar et al. 2007; Medicherla et al. 2007). Therapeutic benefit may be primarily driven by the host immune response against the tumor.

SD-208 also rendered beneficial effects on a model of melanoma bone metastasis (Mohammad et al. 2011). In this study 1205Lu melanoma cells were inoculated into the left cardiac ventricle of nude mice and metastasis formation and dissemination was monitored with and without administration of SD-208 prior to tumor cell

injection. SD-208 treatment prevented the development of osteolytic bone metastases compared with vehicle treated mice. Moreover, in mice where bone metastases did form, the size of the osteolytic lesions was significantly reduced after 4 weeks treatment.

All in all, these results evoke the therapeutic potential of TGF-β inhibitory agents by primarily interfering with TGF-β-mediated immune suppression and, thus, generate a more potent immune response, a result anticipated taking into consideration the phenotype obtained by complete genetic ablation of TGF-β1 in mice.

19.4.5 Possible Side Effects of TGF-β Targeting Agents

All the studies with genetically deficient mice demonstrate that TGF-β signaling pathway is essential during development. Nevertheless, the potential deleterious effects of TGF-β inhibition in adult mice still remain to be determined.

TGF-β systemic inhibition affects the entire tumor microenvironment, from the malignant epithelium to all stromal players. Given the importance of TGF-β in normal tissue homeostasis, broad inhibition is predicted to affect a wide array of normal cell functions. One concern with TGF-β targeting therapies is the potential for detrimental side effects, despite that long-term treatments with TGF-β inhibitors do not seem to significantly alter animal morbidity. In particular, because of the immune-mediated disease and lethality associated with the genetic ablation or inhibition of TGF-β signaling in mice, it was unclear if inhibiting this pathway to treat cancer would be compatible with patient survival when delivered for a sustained period.

In contrast to predictions of severe toxicity stemming from TGF-β signaling inhibition, it has been shown that lifetime exposure to systemic soluble TGF-β1, TGF-β3, or pan-TGF-β neutralizing antibody 1D11 in mouse models does not result in significant adverse effects (Ruzek et al. 2003; Yang et al. 2002; Yingling et al. 2004). The soluble TGF-βRII antagonist SR2F was expressed in mice to determine its impact on tumor growth and long-term effect. No severe toxicities were observed over the lifespan of these SR2F-overexpressing mice (Yang et al. 2002); however, lifelong inhibition of TGF-β at biological levels is sufficient to block experimentally induced or implanted mammary tumors and metastatic dissemination (Lahn et al. 2005; Yang et al. 2002).

Expansion of the hypertrophic and proliferation zones of the physes in the femur and tibia was observed in rats treated with TGF-βRI inhibitors (Lahn et al. 2005). Chondrocytes and chondroid matrix were also increased. The degree of hypertrophy was dose- and time-dependent and only affected young animals, presumably due to active physeal growth. The evident role of TGF-β in remodeling of bone claims for monitoring of bone metabolism in clinical studies with TGF-β inhibitors (Lahn et al. 2005).

The effect on the immune system is more complicated to estimate. In animals deficient for TGF-βRII expression on CD4+ T cells, antigen-activated T-cell

activity is enhanced and tumor growth inhibited (Chen and Wahl 2002; Lahn et al. 2005). However, increasing the activity of antigen-activated T cells by blocking TGF-β can result in autoimmune reactions.

The effect on the tumor vasculature is usually not a common readout for TGF-β inhibition therapeutic benefit. However, multiple are the preclinical reports providing evidence that blocking TGF-β signaling has a direct and indirect effect on tumor angiogenesis. Caution should be taken in VEGF-dependent tumors as type-I TGF-β receptor kinase inhibitors (SB-431542 and LY-2157299) and VEGF ligand availability were shown to synergistically promote blood-vessel formation via integrin 5 (Liu et al. 2009). Similar results were reported on SB-431542 facilitating proliferation and sheet formation of ESC-derived ECs (Watabe et al. 2003). VEGF is typically abundant in hypoxic tumors; hence, this may pose a risk for using TGF-β signaling inhibitors that may potentiate VEGF/VEGFR signaling and undesirably improve the angiogenic response.

19.5 Potential for Combinatorial Therapeutic Studies

The still scarce preclinical and clinical data currently available advocate for an anti-angiogenic and growth inhibitory effect of attenuated ALK1 signaling in cancer, hence sustaining the clinical development of drugs blocking ALK1. Furthermore, while ALK1 targeting monotherapies have been incredibly successful both in preclinical and clinical settings, so far, it is reasonable to anticipate that a combined targeted therapy can hold an agonistic effect fighting cancer disease. The multitude of cancers and heterogeneity within each malignancy combined with case-to-case special demands highly requests for incorporation of two or three different targeted drugs that have independently been beneficial. Future studies should therefore embark on such endeavurs.

Interestingly, VEGF levels are elevated in the aorta, lungs, liver, and intestine of ALK1-deficient mice (Shao et al. 2009), suggesting that double targeting VEGFR and ALK1 signaling pathways may not only be the route to a more efficacious treatment plan but may also circumvent the risk of refractoriness to anti-angiogenic drugs. Of note, bevacizumab was recently reported to attenuate VEGF-induced angiogenesis in ALK1 deletion-induced vascular malformations in the adult mouse brain (Walker et al. 2012).

In a human/mouse chimera tumor model, targeting human ALK1 decreased tumor vessel density and improved antitumor efficacy when combined with bevacizumab (Hu-Lowe et al. 2011). This study raised thus the question whether ALK1 signaling may be part of a set of adaptive mechanisms in tumors refractory to VEGF inhibition (Hu-Lowe et al. 2011). Moreover, ACE-041 used in combination with sunitinib impaired tumor growth in two xenograft models of VEGF-inhibitor resistant renal cell carcinoma, A498 and 786-O. These two models represent surrogates of renal cell carcinoma tumors that typically transiently shrink upon VEGF inhibition but quickly restore angiogenesis and resume tumorigenic program, despite continuation of treatment.

None of the ALK1 targeting studies have so far analyzed the potential adaptive effects of ALK1 targeting drugs in prolonged regimens. In order for ALK1 inhibitors to prevail and make a stand as opposed to VEGF targeting drugs, such analysis is mandatory.

Given the recent studies suggesting a regulatory crosstalk loop amongst BMP9/ALK1 and Notch signaling coordinating tip versus stalk cell specification, one may immediately anticipate possible therapeutic benefit arising from a combinatorial targeting of both pathways in tumor biology.

Neutralization of Dll4-Notch signaling in tumors results in excessive, nonproductive angiogenesis with subsequent inhibitory effects on tumor growth, due to poor perfusion-induced hypoxia (Noguera-Troise et al. 2006; Ridgway et al. 2006). Furthermore, Dll4 has been reported to mediate tumor resistance to bevacizumab in vivo as a compensatory mechanism to VEGF neutralization (Li et al. 2011). Pharmacological targeting of Dll4/Notch signaling in preclinical tumor models has been achieved by several different inhibitory strategies. Specific targeting with anti-Dll4 antibodies does not induce overt toxicity and Dll4 has, thus, emerged as an attractive target for anti-angiogenic cancer therapy (Kuhnert et al. 2011). As Dll4 inhibitors are entering clinical trials for the treatment of solid malignancies, this may pose a novel combinatorial therapeutic opportunity in breast, colon, and renal cancer, where Dll4 is selectively expressed by the endothelium of malignant tissues (Jubb et al. 2006; Jubb et al. 2009; Patel et al. 2005) and where patients may profit from combinatorial actions.

The phase I clinical trial on TRC105 evokes the combinatorial use of drugs impinging their inhibitory effect on both endoglin and VEGFRs, which have shown to generate an ameliorated therapeutic benefit both in the primary tumor burden but also on metastatic dissemination. In fact, a clinical trial analyzing the effect of TRC105 in combination with standard-dose bevacizumab in advanced solid tumors for which bevacizumab is indicated has been launched (www.clinicaltrials.gov). Interestingly, a study reported on a patient with HHT1 who had a substantiated response to bevacizumab (Bose et al. 2009). In this study, the epistaxis episodes became sparser and shorter. In different studies, a patient with HHT1 who received bevacizumab for malignant mesothelioma had a dramatic reduction in gastrointestinal bleeding from arteriovenous malformations (Flieger et al. 2006). Also, a patient with severe hepatic HHT who received six courses of bevacizumab no longer required liver transplantation and was well 6 months after completing the treatment (Mitchell et al. 2008). These studies strongly suggest a strong collaborative action of double targeting simultaneously endoglin and VEGF signaling to reacquire endothelial homeostasis. Furthermore, studies in our laboratory provide evidence for an agonistic effect by using endoglin and VEGFR targeting strategies reducing primary tumor burden and metastatic dissemination (Anderberg et al. 2013).

TGF-β has been reported to promote migration, invasion, and survival in breast cancer cells overexpressing the HER2 oncogene and to accelerate the metastasis of neu-induced mammary tumors in mice (Muraoka-Cook et al. 2005; Muraoka et al. 2003; Siegel et al. 2003). A clearer understanding of the molecular mechanisms underlying the crosstalk between TGF-β and HER2 has started to emerge.

In recent studies the synergistic effect of TGF-β and HER2 on tumor progression has been shown to likely be a combined result of two distinct features: loss of TGF-β tumor suppressive effect and gain of pro-survival and -migratory function through HER2-dependent mechanisms (Chow et al. 2011). In HER2-overexpressing breast cancer, this crosstalk results in increased cancer cell proliferation, survival and invasion, accelerated metastasis, and resistance to chemotherapy and HER2-targeted therapy (Chow et al. 2011). The transformed cellular context stemming from HER2 amplification disrupts the tumor suppressive role of TGF-β and promotes its oncogenic role. In turn, TGF-β potentiates oncogenic HER2 signaling by eliciting shedding of the ERBB ligands and clustering of HER2 with integrins (Wang et al. 2009). Blockade of TGF-β-HER2 crosstalk may suppress breast cancer progression and metastasis and enhance the efficiency of conventional therapies in patients with HER2-overexpressing breast cancer, which afflicts 25–30 % of all breast cancer patients. Moreover, targeting both TGF-β and EGFR/HER2 signaling can represent a stronger action towards the tumor neoendothelium as both have been shown to, among other functions, impair tumor angiogenesis (Izumi et al. 2002). More specifically, Herceptin, a HER2 neutralizing antibody, upregulates TGF-β target genes, such as PAI-1 and Thrombospondin-1 in vivo, rendering a decrease in vessel diameter and in tumor burden (Izumi et al. 2002).

Combinatorial studies on TGF-β inhibition with VEGFR impairment have not yet been established; however, some studies suggest that targeting both pathways may bypass the tumor adaptive actions by means of exploring alternative pathways to maintain tumor growth (Liu et al. 2009).

19.6 Perspective

The pleiotropy and intricacy of TGF-β family signaling conveys effects virtually in all cell types in the body. In cancerous disease, it is well established that TGF-β holds a bipolar role in carcinogenesis, acting as tumor suppressor during the initial stages of tumor development, whilst promoting tumor growth and metastatic spread in advanced stages (Ikushima and Miyazono 2010). As a consequence, the potential of using to our advantage the knowledge on TGF-β biology is still not fully embraced. The development and use of inhibitors of TGF-β family activity for the treatment of cancer may conduct to disparate effects depending on the stage of the disease. Furthermore, in ECs, the overall result of signaling from TGF-β family receptors is manifold and determined by a plethora of factors: ligand specificity or redundancy, engaged type I, type II receptors, and co-receptors (Cunha and Pietras 2011). In spite of the challenges of the elaborated signaling network outcomes from the TGF-β receptors in the various cell types of a tumor, the dividends for modulating the TGF-β network in therapeutic regimens may be rewarding. The current preclinical data and preliminary clinical results readily support the feasibility of using ALK1 and endoglin inhibitors as angiogenesis counteracting agents and TGF-β inhibitors affecting all the tumor cellular compartments.

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