Chapter 1
Transforming Growth Factor-β Signaling

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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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A. Moustakas and K. Miyazawa (eds.), *TGF-β in Human Disease*,
DOI 10.1007/978-4-431-54409-8_1,

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

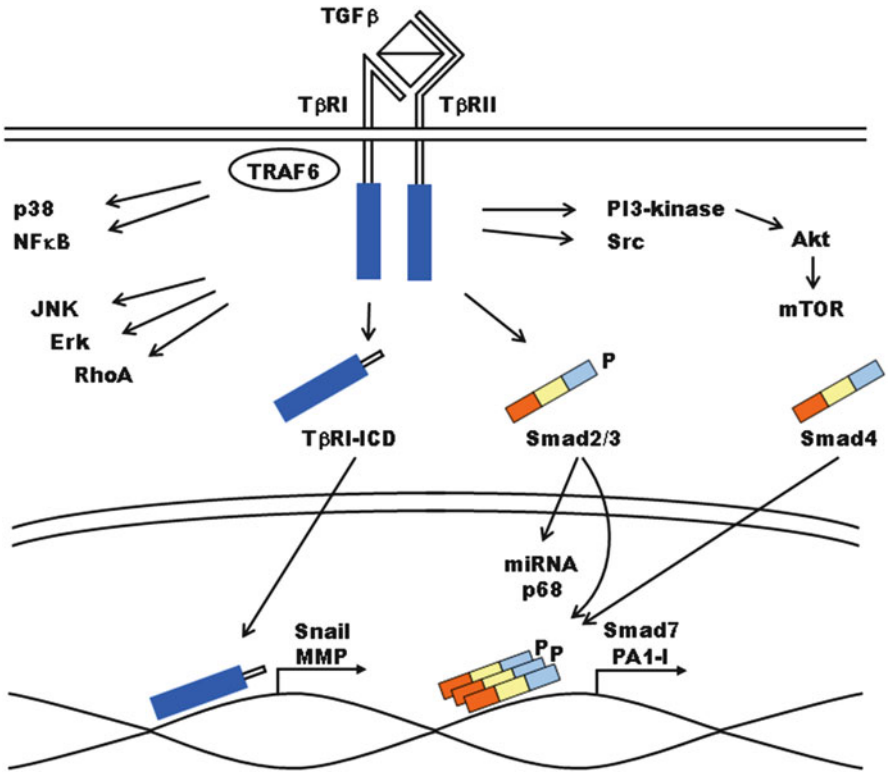

Fig. 1.1 Schematic illustration of major signaling pathways downstream of TGF-β receptors. For explanation, see the text

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

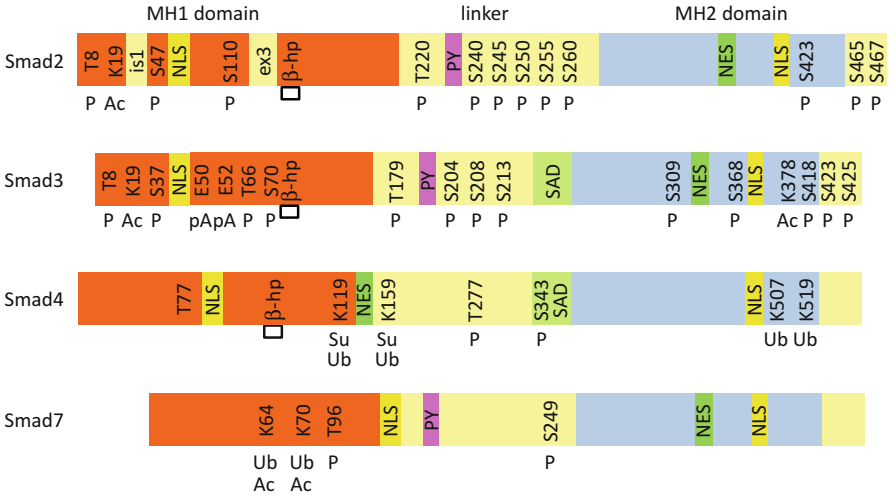

Fig. 1.2 Schematic illustration of the domain structures of the Smad molecules that are involved in TGF-β signaling. Amino acid residues undergoing posttranslationational modifications are indicated. *Is1* inserted sequence 1, *ex3* exon3, *NLS* nuclear localization sequence, *NES* nuclear export sequence, *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.

Acknowledgment Ingegärd Schiller is gratefully acknowledged for valuable help in the preparation of this manuscript.

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