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PROSPECTS

A Tale of Two Proteins: Differential Roles and Regulation of Smad2 and Smad3 in TGF-β Signaling

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Abstract Transforming growth factor-beta (TGF-β) is an important growth inhibitor of epithelial cells, and insensitivity to this cytokine results in uncontrolled cell proliferation and can contribute to tumorigenesis. Smad2 and Smad3 are direct mediators of TGF-β signaling, however little is known about the selective activation of Smad2 versus Smad3. The Smad2 and Smad3 knockout mouse phenotypes and studies comparing Smad2 and Smad3 activation of TGF-β target genes, suggest that Smad2 and Smad3 have distinct roles in TGF-β signaling. The observation that TGF-β inhibits proliferation of Smad3-null mammary gland epithelial cells, whereas Smad3 deficient fibroblasts are only partially growth inhibited, suggests that Smad3 has a different role in epithelial cells and fibroblasts. Herein, the current understanding of Smad2 and Smad3-mediated TGF-β signaling and their relative roles are discussed, in addition to potential mechanisms for the selective activation of Smad2 versus Smad3. Since alterations in the TGF-β signaling pathway play an important role in promoting tumorigenesis and cancer progression, methods for therapeutic targeting of the TGF-β signaling pathway are being pursued. Determining how Smad2 or Smad3 differentially regulate the TGF-β response may translate into developing more effective strategies for cancer therapy. J. Cell. Biochem. 101: 9–33, 2007.

Key words: TGF-β; Smad2; Smad3; transcription factors; localization; cancer; therapeutics

TGF-β SIGNAL TRANSDUCTION

Transforming growth factor-beta (TGF-β) is a member of the TGF-β superfamily of secreted proteins that include bone morphogenic proteins (BMPs) and activins. Specifically, TGF-β is a cytokine involved in immune suppression, angiogenesis, apoptosis, cell growth, and epithelial to mesenchymal transitions (EMT) [Akhurst and Derynck, 2001; Dennler et al., 2002; Moustakas et al., 2002; Schuster and Krieglstein, 2002]. TGF-β signals through the TGFβ type I (TβRI) and TGFβ type II (TβRII) transmembrane serine/threonine protein kinase receptors. When TGF-β binds TβRII, TβRI is recruited to TβRII and forms a heterotetrameric complex [Yamashita et al., 1994]. TβRII is a constitutively active kinase receptor that phosphorylates the glycine-serine rich domain of TβRI, resulting in the activation of TβRI kinase activity [Wieser et al., 1995]. The activated TβRI interacts with and phosphorylates a number of proteins, thereby activating multiple downstream signaling pathways (Fig. 1).

THE SMAD PROTEINS

The TGF-β superfamily directly activates the Smad signaling pathway, in addition to other Smad-independent pathways. Eight mammalian Smad proteins have been identified to date and include Smad1–Smad8. The Smad family of proteins can be divided into three functional groups: the receptor-activated Smads (R-Smads), common mediator Smads (Co-Smads), and the inhibitory Smads (I-Smads; Fig. 2). The R-Smads are directly phosphorylated by the activated type I receptors on their C-terminal

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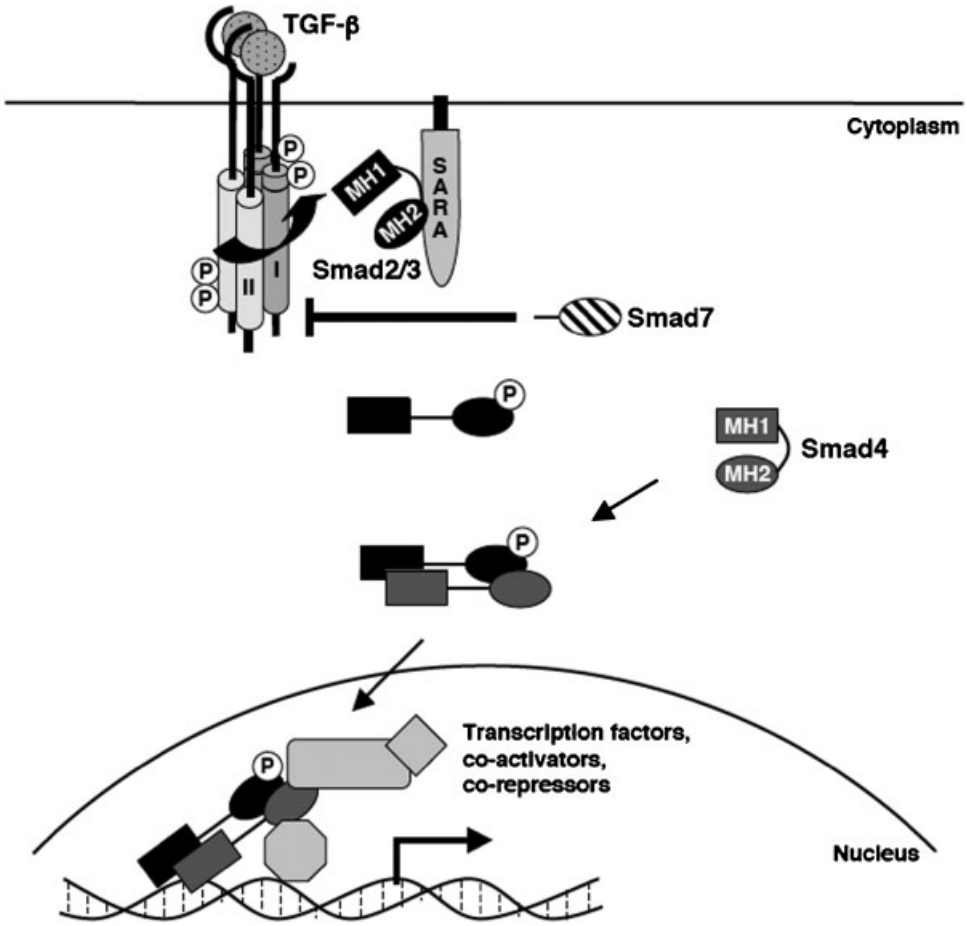

Fig. 1. Activation of TGF-β signaling and the Smad pathway. TGF-β signals through the TGFβ type I (TβRI) and TGFβ type II (TβRII) transmembrane serine/threonine protein kinase receptors. TβRII is a constitutively active serine/threonine kinase receptor that can bind the TGF-β ligand. When TGF-β binds TβRII, TβRI is recruited to TβRII, forming a hetero-tetrameric complex. TβRII phosphorylates TβRI, which results in the activation of the TβRI kinase. The Smad signaling pathway is initiated by phosphorylation of Smad2 and/or Smad3 on their C-termini by TβRI. Adaptor proteins, like Smad anchor for receptor activation (SARA), bind and present Smad2 and Smad3 to the TGF-β receptors. Upon activation, Smad2 and Smad3 oligomerize with Smad4, and translocate to the nucleus where they interact with DNA, transcription factors, coactivators, and/or co-repressors to modulate transcription of target genes. Smad7 negatively regulates TGF-β signaling by competing with Smad2 and Smad3 for TβRI binding.

Ser-Ser-X-Ser (SSXS) motif and include Smad1, Smad2, Smad3, Smad5, and Smad8. Smad2 and Smad3 are phosphorylated in response to TGF-β and activin, whereas Smad1, Smad5, and Smad8 are phosphorylated in response to BMP. This C-terminal phosphorylation allows R-Smad binding to Co-Smads and translocation to the nucleus where they can recruit transcriptional co-activators or co-repressors and regulate TGF-β target genes. The only mammalian Co-Smad identified thus far is Smad4 and it mediates signals from both the TGF-β/activin and BMP signaling pathways. By oligomerizing with activated R-Smads Smad4 participates in signal transduction. The I-Smads, Smad6 and Smad7, are induced by BMP, TGF-β, or activin and act as negative feedback to inhibit activation of the R-Smads by inducing degradation of the receptors or by competing with the R-Smads for TβRI binding.

The Smad proteins are characterized by two conserved regions known as the N-terminal Mad homology domain-1 (MH1) and C-terminal Mad homology domain-2 (MH2), which are joined by a short, poorly conserved linker region (Fig. 2). The MH1 domain is highly conserved among the R-Smads and the Co-Smad, whereas the I-Smads lack an MH1 domain. Smad2 and

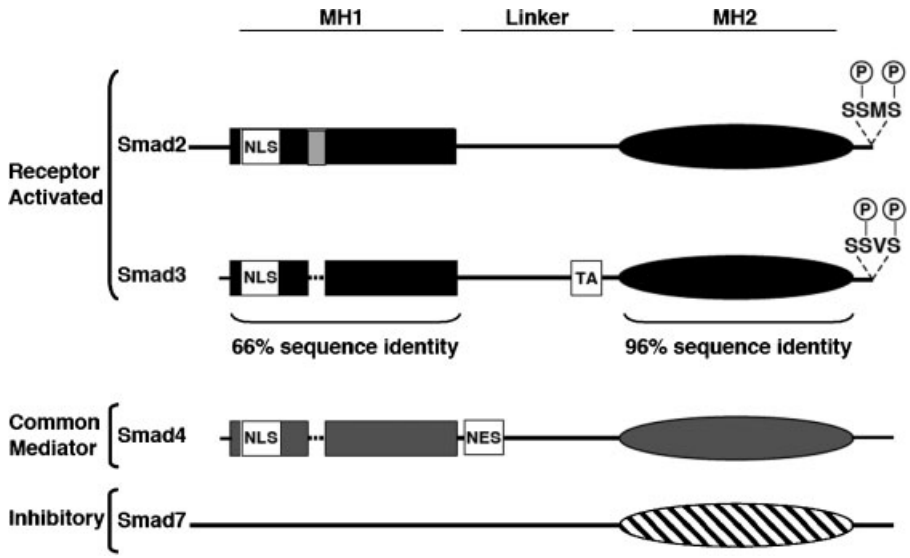

Fig. 2. Smad proteins downstream of the TGF-β signaling pathway. The Smad proteins are characterized by the presence of a Mad homology domain-1 (MH1) and/or a Mad homology domain-2 (MH2). The Smad signaling pathway is initiated by phosphorylation of the receptor activated Smads (Smad2 and/or Smad3) on two C-terminal serine residues (Ser 465/467) by activated TβRI. The MH1 and MH2 domains of Smad2 and Smad3 are highly homologous, however the MH1 domain of Smad2 contains an extra 30 amino acids (gray box) that does not allow Smad2 to bind DNA. The MH1 domains of Smad2, Smad3, and Smad4 contain nuclear localization signals (NLS). The MH1 and MH2 domains are separated by a less-homologous linker region. Smad4 contains a nuclear export signal (NES) and Smad3 contains a transactivation domain (TA) in their linker regions. The common mediator Smad, Smad4, oligomerizes with Smad2 and Smad3 after receptor activation. Smad7 is an inhibitory Smad that lacks the conserved MH1 domain.

Smad3 have 66% amino acid sequence identity between their MH1 domains and 96% amino acid sequence identity between their MH2 domains. The R-Smads and Smad4 have N-terminal nuclear localization signals (NLS) and Smad4 has a nuclear export signal (NES) in the MH1 domain [Xiao et al., 2000a, 2003; Kurisaki et al., 2001]. The MH1 domain plays a role in R- and Co-Smad nuclear import, cytoplasmic anchoring, DNA binding, and regulation of transcription. The MH2 domain is conserved among all of the Smad proteins and regulates Smad oligomerization, cytoplasmic anchoring, and transcription of target genes. The MH1 and MH2 domains bind to a number of proteins including ubiquitination adaptors and substrates, transcriptional co-activators and co-repressors, and a number of transcription factors [Moustakas et al., 2001]. Furthermore, Smad3 has a transactivation domain in the linker region [Prokova et al., 2005]. The functional roles that have been identified for the linker region of the R-Smads are ubiquitination and transcriptional activation [de Caestecker et al., 2000b].

REGULATION OF SMAD SIGNALING BY TGF-β

Receptor Activation of Smad2 and Smad3

SARA and HRS/HGS. The Smad signaling cascade is initiated by C-terminal phosphorylation of Smad2 and/or Smad3 by activated TβRI (Fig. 1) [Macias-Silva et al., 1996]. However, in order for Smad2 and Smad3 to be phosphorylated by TβRI, they must be recruited to the activated receptor complex. A number of proteins have been identified that interact with Smad2 and/or Smad3 to regulate R-Smad phosphorylation. Smad anchor for receptor activation (SARA) and hepatocyte growth factor-regulated tyrosine kinase substrate (HRS/HGS) are FYVE domain containing proteins that present Smad2/3 to TβRI [Tsukazaki et al., 1998; Miura et al., 2000]. SARA is associated with the plasma membrane and can interact with both non-phosphorylated Smad2/3 and the TGF-β receptor complex [Tsukazaki et al., 1998]. When the receptors become activated, and Smad2/3 are phosphorylated, Smad2/3 dissociate from SARA and the receptor complex,

and bind to Smad4. SARA has a higher affinity for monomeric Smads; therefore it is thought that SARA may also act to regulate Smads by inhibiting aberrant Smad2/3 oligomerization [Qin et al., 2002]. Deletion of the FYVE domain in SARA results in the mislocalization of Smad2/3 and the inhibition of TGF-β-dependent transcriptional responses [Tsukazaki et al., 1998]. HRS/HGS is localized to early endosomes and synergizes with SARA to present Smad2/3 to the activated receptor complex [Burd and Emr, 1998; Gaullier et al., 1998; Patki et al., 1998; Miura et al., 2000]. Interestingly, it is observed that Smad2, and to a lesser extent Smad3, binds to SARA in human mesangial cells and this difference in binding may be responsible for the divergence between Smad2 and Smad3 activation and/or TGF-β responses in different cell types [Runyan et al., 2005]. In hepatic stellate cells Smad2 is preferentially activated in early cultured cells, whereas Smad3 is primarily activated in transdifferentiated cells in vitro [Liu et al., 2003]. Furthermore, this transdifferentiation is accompanied by a loss of SARA protein [Liu et al., 2003]. These data are consistent with the idea that SARA availability may control the ability of Smad2 versus Smad3 to be phosphorylated by active TGF-β receptors.

Other cytoplasmic and membrane-bound adaptor proteins. In addition to SARA, Smad2 and Smad3 interact with a number of other cytoplasmic and membrane-bound adaptor proteins that regulate their activation. Cytoplasmic PML (cPML) and the adaptor molecule disabled-2 (DAB-2) physically interact with Smad2 and Smad3 and enable phosphorylation of Smad2 and Smad3 by the activated TGF-β receptor complex [Lin et al., 2004]. cPML also interacts with SARA and is required for the association of Smad2 and Smad3 with SARA [Lin et al., 2004]. In addition, non-phosphorylated Smad2 and Smad3 interact with axin, a negative regulator of Wnt signaling, in the cytoplasm. After TGF-β stimulation Smad3 is targeted to TβRI and is released from axin [Furuhashi et al., 2001]. Smad2 and Smad3 do not interact with axin and SARA in the same complex, and R-Smad association with axin does not affect SARA and Smad3 complex formation [Furuhashi et al., 2001]. A role for axin in Smad2-mediated TGF-β signaling, however, has not been reported. TGF-β receptor-associated protein-1 (TRAP-1), a Smad4 chaperone and inactive TβRI binding protein, facilitates Smad2/4 complex formation upon TGF-β stimulation, however the role of TRAP-1 in Smad3 activation has not been described [Charng et al., 1998; Wurthner et al., 2001].

Smad2 and Smad3 associations with cytoplasmic or membrane-bound proteins may regulate their *differential* activation by the TGF-β receptor complex, and/or Smad2 or Smad3-specific signaling. The adaptor protein embryonic liver fodrin (ELF) interacts with receptor-associated Smad3 and Smad4, and not Smad2, after TGF-β stimulation [Tang et al., 2003]. This interaction facilitates nuclear translocation of Smad3/4 and TGF-β transcriptional responses [Tang et al., 2003]. It is not known if ELF is necessary for TGF-β-induced phosphorylation of Smad3, although it is implied. Modulation of Smad3 or Smad4 at the receptor level, through ELF, may be responsible for activation of specific TGF-β responses. Further studies are needed to identify TGF-β responses that are specifically mediated by Smad3/4. In contrast, TRAP-1-like protein (TLP) differentially regulates TGF-β-induced gene expression by activating Smad2-dependent responses and blocking Smad3-dependent transcription; the latter is accomplished by inhibiting the formation of Smad3 and Smad4 complexes [Felici et al., 2003]. TLP constitutively binds to a subset of TβRIIs, but only interacts with Smad4 in the presence of TGF-β stimulation [Felici et al., 2003]. Induction of an activin response element (ARE)-luciferase reporter was observed in HepG2 cells when TLP was overexpressed. This induction can be attributed to a positive effect of TLP through Smad2 or a negative effect of TLP through Smad3 since Smad2 and Smad3 have opposite activities on the ARE reporter [Piek et al., 2001]. In contrast, overexpression of TLP suppressed TGF-β-induced transcriptional activity of the artificial Smad binding element (SBE_4)-luciferase reporter. The SBE_4-luciferase reporter is only activated by Smad3 and is not activated by Smad2 [Dennler et al., 1999; Yagi et al., 1999]. This differential regulation is carried out by the selective inhibition of Smad3/4 complex formation by TLP. However, TLP does not bind Smad2 or Smad3 and has no effect on R-Smad phosphorylation. Upon Smad2 overexpression and activation of TGF-β signaling, TLP dissociates from Smad4, therefore suggest-

ing that TLP is not in a complex with activated R-Smads and Smad4 [Felici et al., 2003]. Although the differential activation of Smad2 and Smad3 can be a result of interactions with a unique subset of proteins at the TGF-β receptor complex, Smad2 and Smad3 regulation can also result from indirect protein associations.

Smad Nuclear Import

Phosphorylation and oligomerization of Smad proteins. In the non-phosphorylated state, R-Smads exist primarily as cytoplasmic monomers in vivo [Kawabata et al., 1998]. They are auto-inhibited through an interaction between their own MH1 and MH2 domains [Hata et al., 1997]. However, upon phosphorylation on the C-terminal SSXS motif, Smad2 and Smad3 undergo a conformational change that relieves their auto-inhibition, thereby allowing the formation of heteromeric complexes with Smad4. Smad4 also contains a loop in its MH2 domain that prevents oligomerization in the absence of signaling [Tada et al., 1999]. Phosphorylation of Smad2 on Ser^{465} and Ser^{467} is required for oligomerization with Smad4 in mammalian cells [Abdollah et al., 1997; Souchelnytskyi et al., 1997]. Mutation of either one of these serines results in disruption of the Smad2-Smad4 complex, decrease in nuclear accumulation of Smad2 and Smad4, and a decrease in Smad2 transcriptional activity in response to TGF-β1 [Abdollah et al., 1997; Souchelnytskyi et al., 1997]. Mutation of another C-terminal serine, Ser^{464}, also results in disruption of Smad2/Smad4 complexes, although this site is not phosphorylated by activated TβRI [Abdollah et al., 1997]. The exact composition of the heteromeric Smad complexes is controversial as it is debated that the Smad2/Smad4 and Smad3/Smad4 heteromeric complexes are composed of one and one, two and one, one and two, or two and two molecules each of R-Smad and Smad4, respectively [Wu et al., 1997; Kawabata et al., 1998; Chacko et al., 2001; Inman and Hill, 2002]. Additionally, phosphorylation of Smad2 and Smad3 leads to the formation of homo- and hetero-oligomeric complexes with one another [Nakao et al., 1997b; Kawabata et al., 1998]. The resulting R-Smad and Smad4 oligomers can accumulate in the nucleus where they modulate the transcription of many genes (Fig. 1) [Massague, 2000; Massague and Wotton, 2000; de Caestecker et al., 2000a; Ten Dijke et al., 2002].

Smad nuclear localization signal. The mechanisms directing Smad subcellular localization are not fully understood. As mentioned previously, R-Smads contain a N-terminal NLS [Xiao et al., 2000a]. Prior to activation, the NLS is masked and its exposure is triggered by R-Smad phosphorylation by the activated TGF-β receptors [Xiao et al., 2000a,b]. It was thought that R-Smad oligomerization with Smad4 was necessary for R-Smad translocation to the nucleus. Recently, however, it was shown that Smad4 is not required for Smad2 and Smad3 nuclear translocation in a subset of cell lines. In Smad4 deficient breast and pancreatic cancer cell lines, endogenous Smad2 and Smad3 could still localize to the nucleus upon TGF-β1 stimulation [Fink et al., 2003]. In these cell lines, the intrinsic NLS in Smad2 and Smad3 may contribute to their nuclear translocation. Nuclear translocation of Smad2 and Smad3 alone, however, is not sufficient to activate TGF-β-mediated transcriptional responses. These data illustrate the important function of Smad4 in the transcriptional activation/repression of genes.

Smad2 and Smad3 interact with the nuclear pore complex. It is proposed that Smad2 and Smad3 enter the nucleus by overlapping and distinct mechanisms through direct interaction with importin-beta (importin-β) and/or CAN/NUP214 and NUP153, components of the nuclear pore complex (Fig. 3). TGF-β stimulates Smad3 binding to importin-β through its NLS, whereas the putative Smad2 NLS does not bind importin-β [Xiao et al., 2000b; Kurisaki et al., 2001]. Nuclear import of Smad3 requires RAN-GTPase, which mediates the disruption of the Smad3 and importin-β complex in the nucleus [Kurisaki et al., 2001] (Fig. 3A). Although activation of Smad3 by TβRI enhances Smad3 binding to importin-β, it is unknown if this method of nuclear import occurs for Smad3/Smad4 oligomers or for activated monomeric Smad3. In contrast, Smad2 and Smad3 can translocate to the nucleus in an importin-independent manner by associating with CAN/NUP214 and NUP153 via a hydrophobic region in their MH2 domains [Xu et al., 2002] (Fig. 3B). Similarly, TGF-β-activated Smad3/Smad4 complexes are shown to translocate to the nucleus in an importin-independent mechanism [Chen

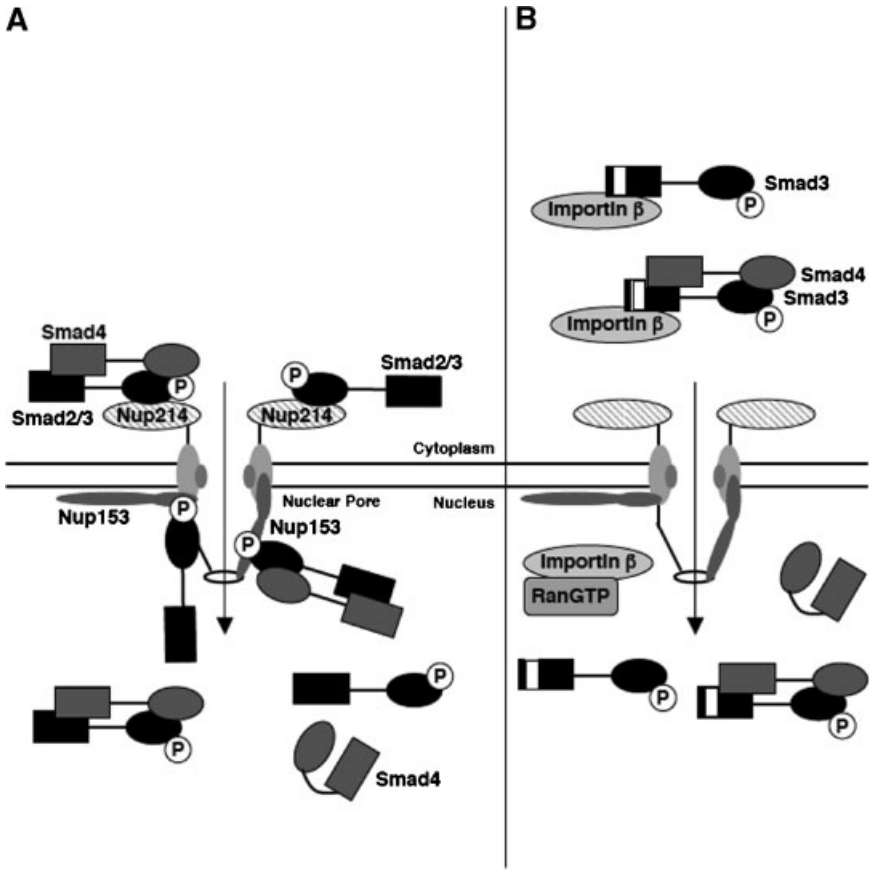

Fig. 3. Mechanisms of Smad2 and Smad3 Nuclear Import. Smad2 and Smad3 enter the nucleus by overlapping and distinct mechanisms by directly interacting with CAN/Nup214 and Nup153 or importin-β. **A**: Phosphorylated Smad2 and Smad3 can directly interact with CAN/Nup214 and Nup153, components of the nuclear pore complex, through a hydrophobic region in the Smad MH2 domains. **B**: Phosphorylated Smad3, but not Smad2, can be translocated to the nucleus through its interaction with importin-β. Smad3 has a NLS in its MH1 domain that binds importin-β whereas the 30 amino acid insert in the MH1 domain of Smad2 inhibits its ability to interact with importin-β. Smad3 nuclear import via importin- is Ran dependent.

et al., 2005]. Within the Smad3/4 oligomer, phosphorylated Smad3, not Smad4, controls nuclear import [Chen et al., 2005]. Consistent with this observation, monomeric Smad4 enters the nucleus utilizing different nucleoporins than the Smad3/Smad4 oligomer [Chen et al., 2005]. In these reports, the nuclear translocation of TGF-β-activated Smad2/Smad4 complexes were not studied, however the region of Smad3 utilized is highly similar to that of Smad2, so it is anticipated that the findings would also apply to the TGF-β-activated Smad2/Smad4 complexes [Chen et al., 2005].

Smad2 and Smad3 translocate to the nucleus by multiple processes; however, it is unclear if selection of these nuclear transport mechanisms is regulated by TGF-β and if there are functional consequences to using one mechanism over another. It is possible that by blocking nuclear transport through CAN/NUP214 and NUP153, Smad3 nuclear import and regulation of TGF-β target genes is selected, over Smad2, via Smad3 interaction with importin-β. Exclusion of Smad2 nuclear accumulation could occur through protein–protein interaction including the MH2 domain of Smad2, thereby inhibiting the ability of Smad2 to interact with CAN/NUP214 and NUP153. The ability of Smad3 to have multiple mechanisms of nuclear import may point to an important role of Smad3 in more immediate TGF-β responses. This idea is consistent with the observation that Smad3 directly activates immediate-early TGF-β target genes, whereas Smad2 primarily regulates immediate-early and intermediate genes through TGF-β and Smad3 [Yang et al., 2003; Piek et al., 2004; Zavadil et al., 2004]. The differential nuclear translocation could be

cell-type dependent or cell-context dependent. Excluding the ability of Smad2 and Smad3 to be phosphorylated by the activated TGF-β receptor complex and to interact with Smad4, there is little evidence suggesting that Smad2 and Smad3 are differentially regulated by TGF-β at the level of nuclear import, although they can be imported through different mechanisms.

Smad Nuclear Export

With prolonged TGF-β stimulation of epithelial cells, Smad2 and Smad3 continuously shuttle between the cytoplasm and the nucleus [Pierreux et al., 2000; Inman et al., 2002; Xu et al., 2002]. The R-Smads that return to the cytoplasm, from the nucleus, are dephosphorylated and are not bound to Smad4 [Pierreux et al., 2000; Inman et al., 2002]. Recently protein phosphatase 1A, magnesium-dependent, alpha/protein phosphatase 2C alpha (PPM1A/PP2Cα) was identified to be responsible for Smad2 and Smad3 dephosphorylation, which results in their disassociation from Smad4 and nuclear export [Lin et al., 2006]. If the receptors remain activated, the dephosphorylated R-Smads are re-phosphorylated and translocate to the nucleus [Inman et al., 2002]. Furthermore, Smad4 has constant nuclear-cytoplasmic shuttling, even in the absence of TGF-β stimulation [Pierreux et al., 2000; Watanabe et al., 2000; Inman et al., 2002]. This constant nuclear and cytoplasmic shuttling of Smad2, Smad3, and Smad4 could be a mechanism for monitoring receptor activity.

Smad2/3 and Smad4 are exported from the nucleus by different mechanisms (Fig. 4). Smad4 has an NLS in the MH1 domain and NES in the linker region [Pierreux et al., 2000; Watanabe et al., 2000] (Fig. 2). This NES binds to chromosome region maintenance 1 (CRM1) and is responsible for Smad4 CRM1-dependent nuclear export [Pierreux et al., 2000; Watanabe et al., 2000] (Fig. 4A). The NES is masked upon complex formation with phosphorylated Smad2 or Smad3 so that it may aid in the regulation of TGF-β target genes [Pierreux et al., 2000]. An NES in the Smad2 or Smad3 proteins has not been characterized and nuclear export of Smad2 has shown to be CRM1-independent [Xu et al., 2002]. Nuclear export of Smad2 and Smad3 is mediated by NUP153 and CAN/NUP214, the same proteins that can regulate their nuclear import [Xu et al., 2002] (Fig. 4B). Non-phosphorlyated, monomeric Smad2 and Smad3 can bind NUP153 and CAN/NUP214 and be transclocated from the nucleus to the cytoplasm, thereby recycling of Smad2 and Smad3 for activation if there is continuous TβRI activation. A mechanism allowing nuclear export of monomeric Smad2, Smad3, and Smad4 may be a means to respond quickly to TGF-β stimulus.

It is unknown whether other nuclear export mechanisms differ between Smad2 and Smad3. Smad4 can bind both Smad2 and Smad3 to regulate TGF-β target genes therefore it is unlikely that accessibility of the Smad4 NES controls Smad2 versus Smad3 signals in the nucleus. Furthermore, it is unknown if more than one mammalian Smad2 and Smad3 phosphatase regulates Smad2 and Smad3 export from the nucleus. Examining Smad2/Smad3 phosphatase specificity could provide important information concerning regulation of Smad2 versus Smad3 nuclear export. Alternatively, there may not be regulation of Smad2 versus Smad3 nuclear export because Smad2 and Smad3 differential activation could be regulated at the level of receptor activation, nuclear import, and/or through other protein-protein interactions in the nucleus.

Negative Regulation of Smad Proteins

In order to prevent continuous Smad signaling in the absence of TGF-β stimulation, Smad2 and Smad3 are negatively regulated by a number of proteins. As previously mentioned, PPM1A/PP2Cα can dephosphorylate activated Smad2 and Smad3 [Lin et al., 2006]. Further, the I-Smad proteins, Smad6 and Smad7, inhibit Smad2/3 activation by competing with Smad2/3 for binding to the TGF-β receptors [Hayashi et al., 1997]. These I-Smads are induced by activation of TGF-β signaling and form a negative feedback loop [Nakao et al., 1997a; Afrakhte et al., 1998; Ishisaki et al., 1998]. The WD40 domain-containing protein, serine-threonine kinase receptor-associated protein (STRAP), recruits Smad7 to activated TβR1 and inhibits TGF-β-induced transcriptional responses. Through this mechanism, STRAP prevents Smad2 and Smad3 access to the receptor complex [Datta et al., 1998; Datta and Moses, 2000]. In addition, the E3 ubiquitin ligases, Smad ubiquitination regulatory factor 2 (SMURF2) and WWP2, interact with phosphorylated Smad2 and Smad3 to promote their ubiquitination and degradation

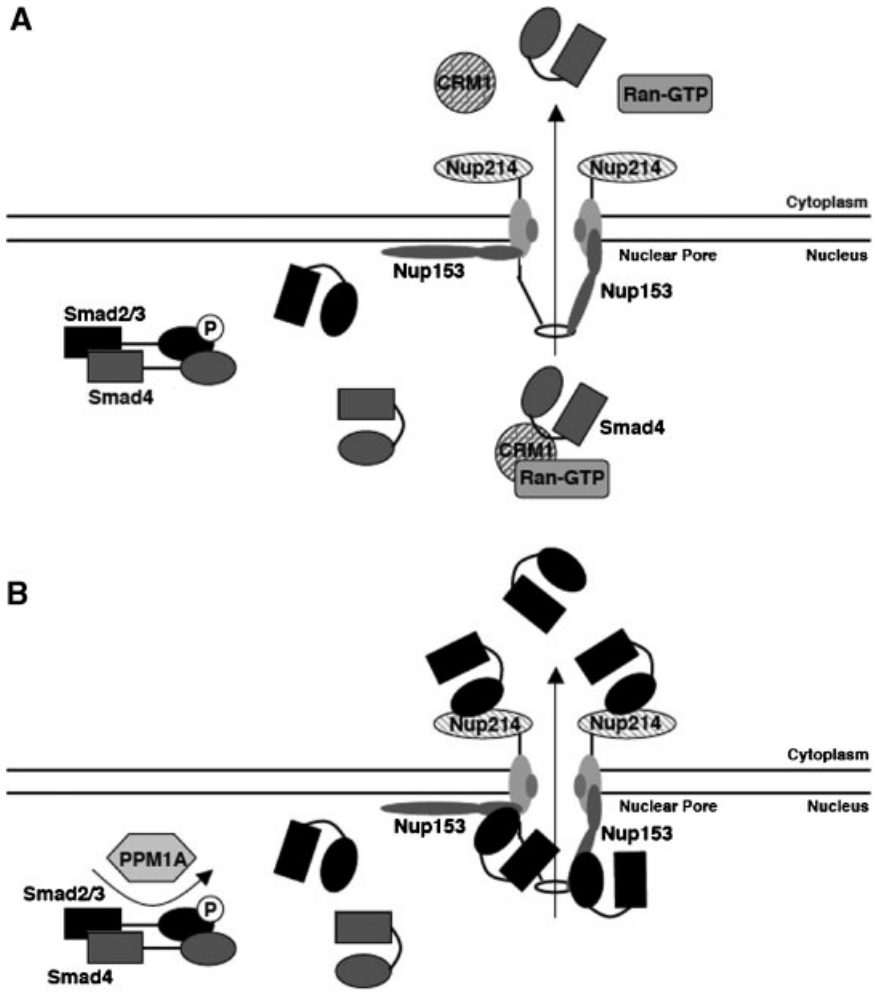

Fig. 4. Mechanisms of Smad2, Smad3, and Smad4 Nuclear Export. Smad2/3 and Smad4 exit the nucleus by distinct mechanisms. **A**: Smad4 nuclear export is mediated by its interaction with CRM1 via a nuclear export signal (NES) in the Smad4 linker region. **B**: Smad2 and Smad3 nuclear export is mediated by CAN/Nup214 and Nup153, in a CRM1-independent manner. After dephosphorylation by PPM1A or another phosphatase, the MH2 domain of unphosphorylated Smad2 and Smad3 bind CAN/Nup214 and Nup153A. CAN/Nup214 and Nup153A are components of the nuclear pore complex that also aid in Smad2/3 nuclear import. Smad4 is not exported through this CAN/Nup214 and Nup153A mechanism. An NES in Smad2 and Smad3 has not been identified.

by the proteasome [Lin et al., 2000; Zhang et al., 2001; Barrios-Rodiles et al., 2005]. Recently, the inner nuclear membrane protein, MAN1, was shown to interact with Smad2 and Smad3 at the inner nuclear membrane in a TGF-β independent manner [Pan et al., 2005]. Overexpression of MAN1 results in inhibition of Smad2 and Smad3 phosphorylation, association with Smad4, nuclear translocation, and repression of activation of the TGF-β target genes [Pan et al., 2005]. Consistent with these data, reduction of MAN1 protein levels enhances TGF-β-mediated responses [Pan et al., 2005]. The above-mentioned mechanisms by which TGF-β signaling is negatively regulated do not discriminate between Smad2 and Smad3.

Evidence exists pointing to differential negative regulation of activated Smad2 and Smad3 by their interactions with E3 ubiquitin ligases. TG-interacting factor (TGIF) interacting ubiquitin ligase 1 (TIUL1) is an E3 ubiquitin ligase that interacts with both Smad2 and Smad3 and induces Smad2 ubiquitination and degradation in the presence of TGIF [Seo et al., 2004]. PRAJA is an E3 ubiquitin ligase that targets Smad3 for degradation [Mishra et al., 2005]. PRAJA expression is high in some gastrointestinal cancers and, therefore, the resulting increase in Smad3 degradation could

be, in part, responsible for uncontrolled cell proliferation [Mishra et al., 2005]. In these studies, the role of PRAJA and TIUL1 in the ubiquitination and degradation of Smad2 and Smad3, respectively, was not determined. Phosphorylated Smad3 is also ubiquitinated by the ROC1, Skp1, Cullin1, and Fbw1a (ROC1-SCF^{Fbw1a}) E3 ligase complex, and is subsequently degraded in the proteasome [Fukuchi et al., 2001; Barrios-Rodiles et al., 2005; Saha et al., 2006]. In contrast, Smad2 is only minimally ubiquitinated by ROC1-SCF^{Fbw1a} [Fukuchi et al., 2001]. The ROC1-SCF^{Fbw1a} complex also assists activated Smad3 nuclear export and its subsequent degradation in the cytoplasm [Fukuchi et al., 2001]. Further characterizing the interactions between Smad2 or Smad3 and TIUL1, PRAJA, and/or ROC1-SCF^{Fbw1a}, or identifying other Smad2 or Smad3 inhibitory proteins may elucidate any selectivity between the regulation of Smad2 and Smad3.

Smad2 and Smad3 can interact with a number of proteins that negatively regulate their activity, however limited evidence suggests that there are distinct mechanisms negatively regulating Smad2 versus Smad3. It is unclear how and if these specific mechanisms are controlled by TGF-β. It is possible that selective negative regulation of Smad2 versus Smad3 would be necessary to turn off one or more TGF-β-mediated Smad response(s) while retaining the other Smad response(s). However, it is uncertain what specific responses are mediated by Smad2 versus Smad3 and, as a result, it is difficult to speculate in what instance one Smad activity is selected over the other.

Transcriptional Control by Smad Proteins

Smad2 and Smad3 DNA binding ability. Once in the nucleus, C-terminally phosphorylated Smad2 and Smad3 regulate the transcription of many genes in concert with a number of co-associated proteins. The Smad3 homomer can form DNA-binding complexes through its MH1 domain independently of Smad4 [Kim et al., 1997; Shi et al., 1998; Dennler et al., 1999]. In contrast, the Smad2 homomer cannot bind DNA without Smad4 because the MH1 domain of Smad2 has an additional 30 amino acids, encoded by exon 3, that prevent direct binding to DNA [Kawabata et al., 1998; Shi et al., 1998; Dennler et al., 1999]. Therefore, Smad2/Smad4 complexes require binding to specific transcription factors to target the complex to DNA [Massague and Wotton, 2000; ten Dijke and Hill, 2004].

Smad-DNA binding specificity. Smad4 and phosphorylated Smad3 bind multiple 5′-AGAC-3′ sequences, called Smad binding elements (SBEs), within the promoters of certain target genes [Kim et al., 1997; Yingling et al., 1997; Dennler et al., 1998; Jonk et al., 1998; Kawabata et al., 1998; Labbe et al., 1998; Shi et al., 1998; Takenoshita et al., 1998; Zawel et al., 1998; Johnson et al., 1999]. Smad3 and Smad4 also bind GC-rich sequences, suggesting that DNA binding specificity of Smad3 and Smad4 is not stringent [Kim et al., 1997; Labbe et al., 1998; Kusanagi et al., 2000; Qing et al., 2000]. Due to the weak interactions of R-Smads with DNA and the high frequency of SBEs in the genome, it is necessary for Smad2 and Smad3 to rely on several common and distinct DNA transcription factors for SBE selectivity and to facilitate transcription of their target genes [Shi et al., 1998; Massague and Wotton, 2000]. Interestingly, SBEs often appear adjacent to binding sites for other transcription factors [Dennler et al., 1998]. In most cases, Smad-binding transcription factors can function independently of Smads to control transcription of specific genes. However, Smad interaction with these transcription factors can modulate their transcriptional activity through recruitment of co-activators or co-repressors.

Regulators of Smad2 and Smad3 transcriptional activity. Smad2 and/or Smad3 have been reported to interact with over fifty known proteins in the nucleus, some of which are listed in Table I (see also [Attisano et al., 2001; Feng and Derynck, 2005]). These Smad2 and Smad3-interacting nuclear proteins include, but are not limited to, transcription factors, transcriptional co-repressors, and transcriptional co-activators that regulate the transcription of TGF-β target genes. The majority of these identified nuclear proteins interact with both Smad2 and Smad3. Smad transactivation of genes can be mediated by binding of the phosphorylated Smad2 and Smad3 with the co-activators p300, cAMP-responsive element-binding protein-binding protein (CBP), and p300/CBP-associated factor (P/CAF) [Feng et al., 1998; Ishisaki et al., 1998; Janknecht et al., 1998; Nishihara et al., 1998; Pouponnot et al., 1998; Shen et al., 1998; Topper

TABLE I. Smad2 and/or Smad3 Nuclear Interacting Proteins

Protein	Function	Smad2	Smad3	References
AP-1 (c-Fos/c-Jun)	Transcription factor	+	+	Zhang et al. [1998], Liberati et al. [1999], Peron et al. [2001], Pessah et al. [2001], Verrecchia et al. [2001a,b]
APC	Ubiquitin ligase	+	+	Stroschein et al. [2001]
AR	Nuclear receptor/transcription factor	−	+	Chipuk et al. [2002]
ATF-2 (CRE-BP1)	Transcription factor	n.d.	+	Sano et al. [1999]
ATF-3	Transcription factor	−	+	Kang et al. [2003]
BRCA1	Nuclear phosphoprotein	+	+	Dubrovska et al. [2005]
BRCA2	Transcriptional regulatory protein	+	+	Preobrazhenska et al. [2002]
c-MYC	Transcription factor	+	+	Feng et al. [2002]
c-SKI	Transcriptional co-repressor	+	+	Akiyoshi et al. [1999], Luo et al. [1999], Xu et al. [2000]
C/EBP α, β, δ	Transcription factors	−	+	Choy and Derynck [2003]
Cited	Transcriptional co-activator	+	+	Chou et al. [2006]
E1A	Adenoviral oncoprotein	+	+	Nishihara et al. [1999]
E2F 4, 5	Transcription factors	n.d.	+	Chen et al. [2002a], Yagi et al. [2002]
EID-2	Transcriptional regulatory protein	+	+	Lee et al. [2004]
ER	Nuclear receptor/transcription factor	+	+	Matsuda et al. [2001]
Evi-1	Transcription factor	+	+	Kurokawa et al. [1998], Alliston et al. [2005]
FAST-1	Transcription factor	+	+[a]	Chen et al. [1997], Liu et al. [1997], Weisberg et al. [1998], Zhou et al. [1998]
FAST-2	Transcription factor	+[a]	+[a]	Labbe et al. [1998], Liu et al. [1999], Nagarajan et al. [1999]
FoxO 1, 3, 4	Transcription factors	−	+	Seoane et al. [2004]
Gli3 (truncated)	Transcription factor	+	+	Liu et al. [1998]
GR	Nuclear receptor/transcription factor	n.d.	+	Song et al. [1999]
HNF4	Orphan nuclear receptor/transcription factor	−	+	Kardassis et al. [2000], Chou et al. [2003]
HOXA13	Transcription factor	+	n.d.	Williams et al. [2005]
LEF1/TCF	Transcription factor	+	+	Labbe et al. [2000]
MAN1	Inner nuclear membrane protein	+	+	Hellemans et al. [2004], Lin et al. [2005], Pan et al. [2005]
MAX	Transcription factor	n.d.	+	Grinberg and Kerppola [2003]
MDMX	Ubiquitin proteasome system protein	n.d.	+	Kadakia et al. [2002]
MEF2A/C	Transcription factors	+	+	Quinn et al. [2001], Liu et al. [2004]
Menin	Transcriptional regulator	−	+	Kaji et al. [2001]
NF-κB	Transcription factor	+	+	DiChiara et al. [2000], Lopez-Rovira et al. [2000]
Notch1 ICD	Transcriptional activator	n.d.	+	Blokzijl et al. [2003]
Notch4 ICD	Transcriptional regulator	+	+	Sun et al. [2005]
p68	RNA helicase	+	+	Warner et al. [2004]
p107	Transcriptional co-repressor	n.d.	+	Chen et al. [2002a]
p300/CBP	Transcriptional co-activator	+	+	Feng et al. [1998], Janknecht et al. [1998], Nishihara et al. [1998], Pouponnot et al. [1998]
P/CAF	Transcriptional co-activator	+	+	Itoh et al. [2000]
PAX8	Transcription factor	n.d.	+	Costamagna et al. [2004]
PIAS3	Transcriptional regulatory protein	+	+	Long et al. [2004]
Runx2 (CBFA1/PEBP2)	Transcription factor	+	+	Hanai et al. [1999], Selvamurugan et al. [2004]
SKIP	Transcriptional co-activator	+	+	Leong et al. [2001]
Smad4	Common mediator Smad	+	+	Lagna et al. [1996], Abdollah et al. [1997], Nakao et al. [1997b], Souchelnytskyi et al. [1997], Wu et al. [1997]
SNON	Transcriptional co-repressor	+	+	Stroschein et al. [1999]
SP1	Transcription factor	+	+	Datta et al. [2000], Feng et al. [2000], Pardali et al. [2000]
SREBP-2	Transcription factor	n.d.	+	Grimsby et al. [2004]
SRF	Transcription factor	+	+	Qiu et al. [2003], Lee et al. [2006]
TFE3	Transcription factor	n.d.	+	Hua et al. [1999]
TGIF	Transcriptional co-repressor	+	+	Wotton et al. [1999]
VDR	Nuclear receptor/transcription factor	−	+	Yanagisawa et al. [1999]
YB-1	Transcription factor	n.d.	+	Higashi et al. [2003]
YY1	Transcriptional co-repressor	+	+	Kurisaki et al. [2003]
ZEB-1 (δEF1)	Transcription factor	+	+	Postigo [2003]
ZEB-2 (SIP1)	Transcription factor	+	+	Verschueren et al. [1999], Postigo [2003]
Znf8	Transcription factor	+[a]	+[a]	Jiao et al. [2002]

n.d., not determined; ICD, intracellular domain.
[a]Not determined for human homologue of FAST-1, FAST-2, or Znf8.

et al., 1998; Itoh et al., 2000]. R-Smad oligomerization with Smad4 is important in this transactivation, due to the presence of a Smad-activation domain (SAD) in Smad4, which allows stronger association with p300/CBP co-activators [de Caestecker et al., 2000b; Chacko et al., 2001]. p300, CBP, and P/CAF have histone acetyltransferase (HAT) activity, which facilitates Smad contact with DNA and with other transcriptional machinery [Ten Dijke et al., 2002]. Smad2 and Smad3 can also indirectly associate with histone deacetylases (HDACs) mediated by other proteins that recruit HDACs, such as SIN3A [Liberati et al., 2001]. TGIF is a co-repressor that binds Smad2 and Smad3 and recruits mSin3, forming a transcriptional repressor complex [Wotton et al., 1999, 2001]. The SKI and SNON proteins can also bind activated Smad2 and Smad3 and act as transcriptional co-repressors [Luo et al., 1999; Stroschein et al., 1999; Xu et al., 2000]. SKI and SNON can recruit HDACs by binding to nuclear receptor co-repressor (N-CoR) and Sin3A [Akiyoshi et al., 1999; Luo et al., 1999; Stroschein et al., 1999; Sun et al., 1999]. Similar to TGIF, SKI can also compete with p300 for Smad binding and disrupt the functional R-Smad/Co-Smad complexes [Akiyoshi et al., 1999; Wu et al., 2002]. SNON binds Smad2 and Smad4, and only small amounts of SNON bind Smad3 [Stroschein et al., 1999]. SKI and SNON are, however, rapidly degraded in response to TGF-β signaling, allowing Smad-dependent regulation of TGF-β target genes [Sun et al., 1999]. The transcription factor SRF was shown to inhibit TGF-β signaling through interaction with Smad2 and Smad3 by inhibiting the DNA-binding activity of the Smad3/Smad4 complex [Lee et al., 2006]. SP1 interacts with both Smad2 and Smad3 and plays a part in the activation of *p21* transcription [Pardali et al., 2000]. The above-mentioned nuclear proteins interact with both Smad2 and Smad3 and do not selectively regulate Smad2 versus Smad3 transcriptional responses.

Balancing Smad2 and Smad3 transcriptional regulation. Although Smad2 and Smad3 are capable of interacting with a number of common transcriptional regulators, there is evidence to suggest that some of these proteins act to alter the balance of Smad2 versus Smad3-mediated TGF-β signaling in the nucleus. The abundance of some of the interactions between Smad2 and/or Smad3 and their nuclear interacting proteins differ and many of the proteins have been shown to only cooperate with one Smad protein, over another, to regulate transcription of target genes. For example, the E1A-like inhibitor of differentiation (EID-2) interacts with both Smad2 and Smad3 and attenuates TGF-β-mediated up-regulation of p21 and p15 [Lee et al., 2004]. However, the interaction between EID-2 and Smad3 is stronger than that of EID-2 and Smad2 and overexpression of EID-2 inhibits endogenous Smad3/4, and not Smad2/4, complex formation, suggesting that EID-2 inhibits Smad3-specific signals in vivo [Lee et al., 2004]. The transcription factor ectopic viral integration site 1 (EVI-1) also interacts with both Smad2 and Smad3 with the interaction between Smad3 and EVI-1 being greater than that between Smad2 and EVI-1 [Kurokawa et al., 1998; Alliston et al., 2005]. However, EVI-1 represses only Smad3-mediated TGF-β signaling and antagonizes the growth inhibitory effects of TGF-β by recruiting the co-repressor CtBP [Kurokawa et al., 1998; Izutsu et al., 2001]. In contrast, EVI-1 represses Smad2-mediated Activin signaling [Alliston et al., 2005]. The functional differences of these protein–protein interactions may be cell type and/or cell signaling dependent.

It is possible that Smad2 and Smad3 are differentially regulated in the nucleus by their interaction with a unique subset of transcription factors to regulate a different subset(s) of target genes. Smad3, but not Smad2, binds the forkhead box (Fox) transcription factors FoxO1, FoxO3, and FoxO4 in a TGF-β dependent manner to activate transcription of *p21* [Seoane et al., 2004]. Loss of FoxO results in the inability of TGF-β1 to activate *p21* in vivo [Seoane et al., 2004]. Smad3 also recruits another transcription factor, activating transcription factor 3 (ATF3), to the *inhibitor of DNA binding 1* (*ID1*) promoter where it mediates repression of *ID1* transcription in response to TGF-β1 [Kang et al., 2003]. ID1 is a protein that promotes cell proliferation and prevents differentiation, therefore repression of *ID1* by TGF-β1 contributes to TGF-β-mediated growth arrest. ATF3 uniquely interacts with Smad3, not Smad2, and overexpression of ATF3 inhibits Smad3 export from the nucleus to the cytoplasm [Kang et al., 2003]. Smad3, not Smad2, interacts with menin to promote Smad3-induced transcriptional activity by aiding in the formation of Smad3/4 complexes with DNA [Kaji et al., 2001]. Menin is

a nuclear protein encoded by *multiple endocrine neoplasia type 1* (*MEN1*), a gene that is sometimes mutated in endocrine tumors of the parathyroid, pancreas, and anterior pituitary [Agarwal et al., 2005]. One mechanism for tumorigenesis arising from mutations in *MEN1* could be the inactivation of the cytostatic response to TGF-β through loss of Smad3-mediated transcription of target genes [Kaji et al., 2001]. The CCAAT/enhancer-binding proteins (C/EBP) α, β, δ also selectively interact with Smad3, and not Smad2, which results in repression of C/EBP transactivation function [Choy and Derynck, 2003]. C/EBPs are transcription factors that promote adipocyte differentiation [Rosen, 2005]. TGF-β, an inhibitor of adipocyte differentiation, enhances this Smad and C/EPB interaction and suppression of C/EBP transactivation of target genes [Choy and Derynck, 2003]. Many of these Smad3-interacting transcription factors are ubiquitously expressed and Smad3 interactions with these proteins may be a common mechanism for regulation of Smad3-specific transcriptional responses.

In addition to transcription factors, Smad3 can also uniquely interact with a number of nuclear receptors that act as transcription factors such as the vitamin D receptor (VDR), the androgen receptor (AR), and hepatocyte nuclear factor-4 (HNF4). The VDR and Smad3 interaction is driven by the VDR ligand 1,25-dihydroxyvitamin D_3 (vitamin D), a hormone involved in calcium and phosphorus metabolism in addition to cell growth and differentiation in various cell types and tissues [Yanagisawa et al., 1999; Gurlek et al., 2002]. Overexpression of Smad3, but not Smad2, stimulates the transactivation function of VDR [Yanagisawa et al., 1999]. In addition, TGF-β1 and vitamin D synergistic activation of the osteocalcin promoter is mediated by VDR but also requires Smad3 [Subramaniam et al., 2001]. Furthermore, Smad7 abrogates Smad3-mediated VDR function, indicating that activation of Smad3 by the TGF-β receptor complex is important for VDR transactivation of target genes as an interaction between Smad7 and VDR was not detected [Yanagi et al., 1999]. Similarly, the orphan nuclear receptor HNF4 interacts with Smad3 to activate the *liver-specific apolipoprotein CIII* (*APOCIII*) promoter in response to TGF-β1 [Kardassis et al., 2000; Chou et al., 2003]. In contrast, Smad2 does not bind to HNF4 and overexpression of Smad2 does not activate *APOCIII* [Kardassis et al., 2000]. Furthermore, Smad3 binding to the HNF response element in the *APOCIII* promoter is not necessary for transactivation of the promoter [Kardassis et al., 2000; Chou et al., 2003]. APOCIII is involved with binding of lipoproteins to cell receptors and is inhibited by a number of pre-inflammatory cytokines, therefore this activation is consistent with the activity of TGF-β as an anti-inflammatory cytokine [Lacorte et al., 1997; Letterio and Roberts, 1998; Kardassis et al., 2000]. In response to 5α-dihydrotestosterone (DHT), AR interacts with Smad3, not Smad2, to repress TGF-β-mediated activation of target gene promoters and expression of TGF-β1, c-Fos, and early growth response gene-1 (EGR-1) in the prostate by inhibiting Smad3 binding to SBEs [Chipuk et al., 2002]. Interestingly, Smad3 can act as a co-regulator to enhance AR-mediated transactivation in the presence of DHT [Kang et al., 2001]. With respect to the tumor suppressor function of TGF-β, these data are consistent with the finding that androgens can promote viability of prostate epithelial cells by preventing TGF-β-induced cell cycle arrest and/or apoptosis. Many of these nuclear receptors are expressed in a tissue-specific manner, therefore these Smad3-specific interactions with nuclear receptors are not ubiquitous and may be unique to certain cell/tissue types.

None of the nuclear proteins listed in Table I were shown to interact with and selectively regulate Smad2-mediated transcriptional responses. Perhaps the inability of Smad2 to bind these specific proteins is due to the presence of exon 3 in the MH1 domain that restricts the binding of Smad2 to DNA. Evidence supporting this hypothesis is that a Smad2 splice variant lacking this insert binds FoxO proteins, whereas the full-length protein does not interact with FoxO [Seoane et al., 2004]. Interactions between Smad3 and HNF4 and VDR are mapped to the Smad3 MH1 domain, however it is unknown if the same Smad2 splice variant lacking the MH1 insert would allow a Smad2 interaction with HNF4 and/or VDR [Yanagisawa et al., 1999; Chou et al., 2003]. In contrast, some of the Smad3-specific nuclear protein–protein interactions that are listed in Table I are not mediated by the MH1 domain, rather they occur through the MH2 domain of Smad3. These proteins include

AR, ATF-3, C/EBP (α, β, δ), and menin [Chipuk et al., 2002; Choy and Derynck, 2003; Kang et al., 2003]. In addition, a few of the proteins listed in Table I interact with the MH1 domain of both Smad2 and Smad3. These proteins include BRCA2 and c-MYC [Pardali et al., 2000; Feng et al., 2002; Preobrazhenska et al., 2002]. Interestingly BRCA2 and c-MYC interact with Smad2 and Smad3 via the MH1 and MH2 domains, independent of the other domain [Feng et al., 2002; Preobrazhenska et al., 2002]. The MH2 domains of Smad2 and Smad3 are highly homologous and one might hypothesize that the more divergent MH1 domain would be responsible for these protein–protein interaction differences between Smad2 and Smad3. However, it has been observed that Smad2 and Smad3 can interact with the same proteins through the MH1 domain and it is unknown what confers protein-binding specificity.

SMAD2 VERSUS SMAD3-MEDIATED TGF-β SIGNALING

Smad2 and Smad3 Knockout Mice Phenotypes

Although the mechanisms by which Smad2 versus Smad3 activation are not fully understood, the phenotypes of the Smad2 and Smad3 knockout mice demonstrate that the proteins have differential roles during embryonic development. Knockout of the Smad2 gene in mice results in early embryonic lethality at embryonic day 7.5–12.5 [Nomura and Li, 1998; Waldrip et al., 1998; Weinstein et al., 1998; Heyer et al., 1999]. This lethality is attributed to the restriction of the site of primitive streak formation and the failure to establish an anterior–posterior axis within the epiblast or formation of the ectoderm, mesoderm, and endoderm [Waldrip et al., 1998; Weinstein et al., 1998; Heyer et al., 1999]. In contrast, knockout of the Smad3 gene in mice is not embryonic lethal [Zhu et al., 1998; Datto et al., 1999; Yang et al., 1999]. In one knockout model, the mice die between 1 and 8 months due to compromised immune function and the formation of colorectal adenocarcinomas [Zhu et al., 1998]. These colorectal tumors are invasive, metastasize to the lymph nodes, and are the cause of death in 100% of the animals by 30 weeks of age [Zhu et al., 1998]. In contrast, these adenocarcinomas were not detected in Smad3 null mice generated by two other groups [Datto et al., 1999; Yang et al., 1999]. Recently it was shown that infection of Smad3 null mice with *Helicobacter pylori* resulted in chronic inflammation and colon cancer in these animals [Maggio-Price et al., 2006]. Thymic involution, enlarged lymph nodes, and formation of bacterial abscesses adjacent to mucosal surfaces are common in Smad3 null mice [Yang et al., 1999]. Smad3-null T cells are constitutively active and are not inhibited by TGF-β1 in vitro [Yang et al., 1999]. Smad3 deficient neutrophils are also impaired in their chemotactic response toward TGF-β [Yang et al., 1999]. The null mice also exhibit forelimb malformations and exhibit accelerated wound healing characterized by an increased rate of re-epithelialization and reduced inflammation [Ashcroft et al., 1999; Datto et al., 1999]. Interestingly, Smad3 deficient mice are protected against cutaneous injury induced by ionizing radiation [Flanders et al., 2002]. A summary of the Smad2 and Smad3 mouse knockout phenotypes are shown in Table II.

Smad2−/− and Smad3−/− Mouse Embryonic Fibroblasts

Mouse embryonic fibroblasts (MEFs) derived from Smad2 or Smad3 deficient mice show differential effects in the activation of multiple Smad reporters and of genes involved in the Smad positive and negative feedback loops. Activation of a luciferase reporter with four repeats of the Smad binding element (SBE_4-luc) is dependent on expression Smad3 but not Smad2, whereas activation of a luciferase reporter that contains an ARE from the *Xenopus Mix.2* gene promoter (ARE-luciferase) is strongly suppressed in Smad2 null MEFs, whereas it is enhanced in Smad3 null MEFs [Piek et al., 2001]. In the Smad3 null MEFs, TGF-β-induction of *c-Fos* is compromised, whereas *c-Fos* is upregulated in Smad2 null MEFs in response to TGF-β [Piek et al., 2001]. *Smad7* and *TGF-β1* autoinduction is also impaired in Smad3 null MEFs and not in Smad2 null MEFs [Piek et al., 2001]. In addition, TGF-β does not induce expression of *Pai-1* or *p15* in the Smad2 and Smad3 deficient MEFs [Datto et al., 1999; Piek et al., 2001]. In contrast, these genes are induced in WT MEFs.

Microarray analysis of Smad2 and Smad3 null MEFs suggests that Smad3, not Smad2, is the essential mediator of TGF-β signaling. Smad3 directly activates immediate-early genes encoding regulators of transcription and signal transducers through Smad3/4

TABLE II. Smad2 and Smad3 Knockout Mouse Phenotypes

Geneotype	Phenotype	References
Smad2−/−	Embryonic lethal E7.5−E12.5[a]	Heyer et al. [1999]
	No formation of head fold or primitive streak	Nomura and Li [1998]
	No development of mesodermal cells	Waldrip et al. [1998]
	No gastrulation	Weinstein et al. [1998]
Smad3−/−	Viable and fertile	Zhu et al. [1998], Datto et al. [1999], Yang et al. [1999]
	Smaller than WT littermates	
	Malformed forelimbs	
	Impaired immune function	
	Chronic intestinal inflammation	
	Colorectal adenocarcinoma (4−6 mo)[b]	
	Accelerated wound healing	Ashcroft et al. [1999]
	Protection from IR	Flanders et al. [2002]

WT, wild-type; IR, ionizing radiation.
[a]Embryonic day 7.5−12.5 (E7.5−E12.5).
[b]Adenocarcinomas were not detected in Smad3 null mice generated by Datto et al. [1999] or Yang et al. [1999] in the same genetic background. Maggio-Price et al. [2006] showed that infection with *Helicobacter pylori* caused chronic inflammation and colon cancer in these animals.

DNA-binding motifs, whereas Smad2 regulates TGF-β-responsive immediate-early and intermediate genes through Smad3 [Yang et al., 2003]. Within 4 h TGF-β1 treatment, the number of genes that were regulated in the Smad3 null MEFs decreased about 17-fold, compared to the WT MEFs. In contrast, the number of genes that were regulated in the Smad2 null MEFs did not decrease compared to the WT MEFs. These results suggest a model in which TGF-β receptors directly activate Smad3 and that Smad2 merely transmodulates the signals.

Knockout of Smad2 or Smad3 in MEFs results in only weak growth inhibition by TGF-β in culture, compared to wild-type (WT) fibroblasts which are growth inhibited by TGF-β [Datto et al., 1999; Piek et al., 2001]. Primary keratinocytes derived from Smad3 null mice also have reduced sensitivity to TGF-β-mediated growth inhibition [Ashcroft et al., 1999]. Although Smad3 is not necessary for TGF-β-mediated growth inhibition in primary unstimulated splenocytes, Smad3 is required for TGF-β-mediated growth inhibition of αCD3-stimulated splenocytes [Datto et al., 1999]. TGF-β-repression of Rb phosphorylation and Cdk2 kinase activity are inhibited in αCD3-activated splenocytes derived from Smad3 null mice compared to those derived from WT mice [Datto et al., 1999]. The reduction of TGF-β sensitivity in the Smad2 and Smad3 deficient MEFs can be attributed the delay in *p15* upregulation and the failure to downregulate *c-Myc* by TGF-β [Piek et al., 2001; Vijayachandra et al., 2003]. In addition, levels of *p21* mRNA are constitutively high, regardless of TGF-β treatment, in the Smad2 and Smad3 null MEFs [Piek et al., 2001].

In contrast to the observation that TGF-β partially inhibits Smad3 null MEF proliferation, TGF-β induces growth inhibition in Smad3 null mammary gland epithelial cells in culture [Datto et al., 1999; Piek et al., 2001; Yang et al., 2002; Vijayachandra et al., 2003]. Compensatory changes in protein levels or phosphorylation of Smad2 are not seen in the Smad3 null epithelium [Yang et al., 2002]. In contrast, TGF-β treatment of murine hepatocytes with conditional deletion of Smad2 results in TGF-β-mediated G1 arrest, apoptosis, and EMT but not in hepatocytes derived from Smad3−/− mice [Ju et al., 2006]. This evidence indicates that Smad3 is not necessary for the growth inhibitory effect of TGF-β in mouse mammary gland epithelial cells but is necessary for this effect and others in mouse hepatocytes. However, it is not known if Smad2 is necessary for the growth inhibitory effects of TGF-β in mammary gland epithelial cells. The different cell types may explain the variation in the effect of TGF-β on cell proliferation (Fig. 5).

Modulating Smad2 or Smad3 Availability Reveals Distinct Functions In Vitro

In addition to the studies perfomed in MEFs, the relative functions of Smad2 and Smad3 in TGF-β signaling is illustrated by RNAi-mediated silencing of Smad2 or Smad3, blocking Smad2 or Smad3 signaling through use of a dominant-negative protein, or by overexpression studies. In HaCaT cells, silencing of Smad2 and

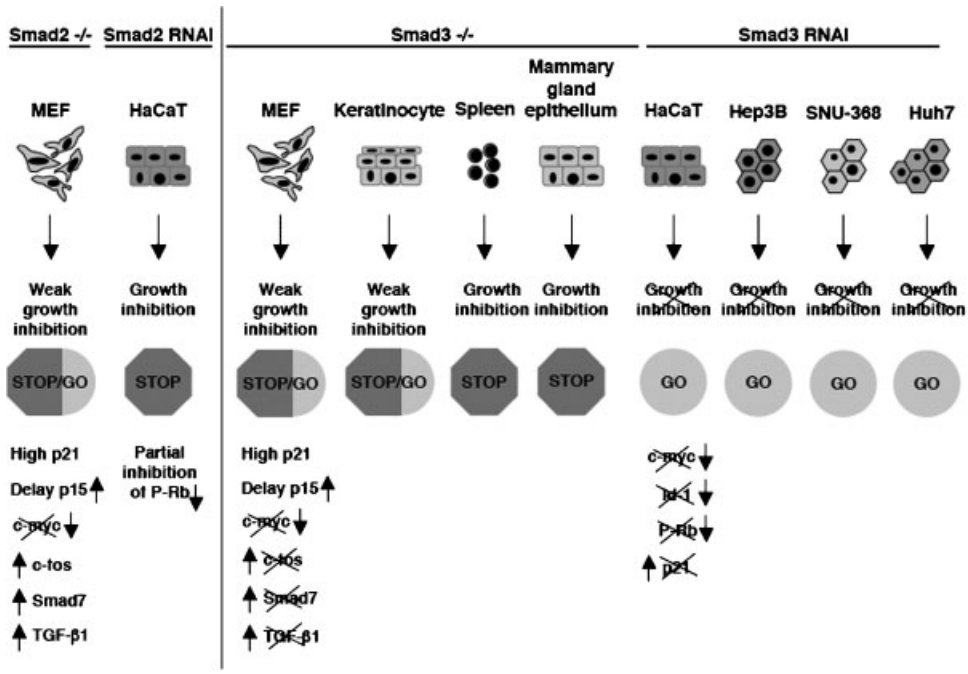

Fig. 5. The effect of Smad2 or Smad3 deficiency on TGF-β-mediated cell cycle control in different cell types. Different cell types exhibit diverse responses to TGF-β when lacking Smad2 or Smad3. Mouse embryonic fibroblasts (MEFs) derived from Smad2−/− or Smad3−/− mice are only partially resistant to the growth inhibitory effect of TGF-β. This effect may be due, in part, to inhibition of c-myc repression, delay in p15 upregulation, and constitutive high levels of p21. Keratinocytes from Smad3−/− mice are also resistant to the growth inhibitory effect of TGF-β whereas splenocytes (spleen) and mammary gland epithelial cells from the same mice are sensitive to TGF-β. In contrast, silencing Smad3 in human keratinocytes (HaCaT) by RNA interference (RNAi) inhibits the growth inhibitory effect of TGF-β whereas silencing Smad2 has no effect on the cell cycle response. The inability of the HaCaT cells deficient in Smad3 to activate p21 and to repress c-myc, Id-1, and Rb phosphorylation may be responsible for their inability to undergo cell cycle arrest in response to TGF-β. Silencing Smad3 in human hepatoma cell lines (Hep3B, SNU-368, Huh7) also results in the inability of TGF-β to induce growth arrest.

Smad3 reduces the TGF-β1-mediated increase of heme oxygenase-1 (HO-1) [Kretschmer et al., 2003]. Silencing of Smad3 diminishes the increase in type 2 transglutaminase (TGase2) and p21 protein levels, and inhibits the decrease of ID1, phosphorylated Rb, and MYC protein levels after TGF-β1 treatment. In contrast, silencing of Smad2 only partially inhibits the reduction of phosphorylated Rb and has no effect on the protein levels of TGase2, p21, ID1, and MYC after TGF-β1 treatment. Interestingly, silencing of Smad3, but not silencing of Smad2, blocks the growth inhibitory response of TGF-β in HaCaT cells indicating that Smad3 may have a more important role in TGF-β-mediated cell cycle arrest than Smad2 (Fig. 5). Microarray analysis of mRNA from HaCaT cells with silenced Smad2 or Smad3 and treated with TGF-β1 identified subsets of genes for which regulation was dependent on Smad2, dependent on Smad3, dependent on Smad2 and Smad3, and not dependent on either Smad2 or Smad3. These studies support the hypothesis that Smad2 and Smad3 have distinct roles in TGF-β signaling.

The cytostatic function of Smad3 over Smad2 in TGF-β signaling was also revealed in another study using gene silencing [Kim et al., 2005]. Smad3 silencing by RNAi resulted in inhibition of TGF-β-mediated cell cycle arrest in a number of TGF-β sensitive cell lines. In addition, raising the relative endogenous ratio of Smad3 to Smad2 by depleting Smad2 enhanced the TGF-β cytostatic response. Interestingly, Smad3 activation and transcriptional activity upon TGF-β1 treatment of cells was facilitated when Smad2 was silenced. For instance, repression of *c-myc* and induction of *p15* and *p21*, by TGF-β1, was greater in HaCaT cells when Smad2 was depleted (Fig. 5). The specific role of Smad3 in TGF-β-mediated growth arrest can also be partially explained by repression of *ID1* through the recruitment of ATF3 [Kang et al., 2003].

The roles of Smad2 versus Smad3 were recently characterized in primary hepatic stellate cells (HSCs) by expressing WT and dominant negative Smad2 and Smad3 [Uemura et al., 2005]. Cells that overexpress Smad3 have increased secretion of fibronectin and type I collagen, increased chemotaxis, decreased proliferation, more focal adhesions, and increased alpha-smooth muscle actin organization in actin stress fibers, compared to cells overexpressing Smad2. These studies further characterize the importance on Smad3 in HSC morphological maintenance.

Evidence in a number of other cell types suggests that Smad2 and Smad3 differentially regulate the transcription of TGF-β target genes. In HepG2 human hepatoma cells, overexpression of the Smad3/4 complex causes higher levels of transcriptional activation of the *p21* promoter than overexpression of the Smad2/4 complex [Moustakas and Kardassis, 1998]. TGF-β induction of *GADD45β* requires Smad3 and Smad4 but not Smad2 [Major and Jones, 2004]. Conversely, the Smad2/4 complex associates with the mammalian forkhead domain protein (FAST-2) to induce the *goosecoid* (*gsc*) promoter, whereas the Smad3/4 complex represses the *gsc* promoter. Repression of the promoter occurs even in the presence of Smad2 and Smad4 when increasing amount of Smad3 are transfected into the cells [Labbe et al., 1998]. In C2C12 myotubes, Smad2 and Smad3 interact with myocyte enhancer-binding factor 2 (MEF2) a transcription factor that regulates gene expression in cardiac and skeletal muscle and plays a role in differentiation and apoptosis in neurons and T cells [Quinn et al., 2001]. In combination with TGF-β1, overexpression of Smad2 activates MEF2 whereas overexpression of Smad3 represses MEF2 transcription [Quinn et al., 2001; Liu et al., 2004]. In addition, it was shown that TGF-β differentially activates Smad2 and Smad3 in hepatic stellate cells [Liu et al., 2003]. Smad2 is phosphorylated in the early passage cells and as these cells differentiate, Smad2 becomes constitutively activated. However, Smad3 is only activated as the cells transdifferentiate [Liu et al., 2003]. Furthermore, in human lung epithelial cells, the expression of Smad3, but not Smad2, is downregulated by TGF-β and overexpression of Smad3 induced apoptosis whereas Smad2 did not induce apoptosis to the same extent [Yanagisawa et al., 1998].

These data above taken together with the different phenotypes of Smad2 and Smad3 deficient mice and the differential regulation of TGF-β target genes in Smad2 and Smad3 deficient MEFs and HaCaT cells, suggest that Smad2 and Smad3 do not only have compensatory roles. However, few experiments have been performed in human epithelial cells. Since most cancers arise from epithelial cells, it would be more informative examine the roles of Smad2 and Smad3 in TGF-β signaling in epithelial cells. Attempts have been made to investigate the role of Smad3 in the mouse mammary gland, however the relative contribution of Smad2 to TGF-β signaling has not been performed in primary Smad2 deficient epithelial cells.

THE SMAD PATHWAY AS A THERAPEUTIC TARGET

Resistance to the anti-proliferative effects of TGF-β is observed in a number of different human cancers by mutations in or transcriptional repression of the genes encoding TβRI, TβRII, Smad2, and/or Smad4 [Levy and Hill, 2006]. Studies show that Smad3 plays an important role in mediating TGF-β signals and, therefore, it is surprising that Smad3 mutations have not yet been detected in human tumors. Although Smad3 mutations have yet to be identified in cancer, loss of Smad3 expression has been identified in gastric cancer [Han et al., 2004]. Loss of Smad3 expression may be due to post-translational modifications as Smad3 can be phosphorylated by CDK4 and CDK2 hindering its ability to transactivate *p15*, repress *c-myc*, and inhibit cell proliferation [Matsuura et al., 2004]. Furthermore, when Smad3 null keratinocytes, transduced with v-ras^{Ha}, are transplanted in nude mice, they rapidly convert from benign papillomas to malignant carcinomas, whereas v-ras^{Ha}-transduced WT keratinocytes only form benign papillomas [Vijayachandra et al., 2003]. These data suggest that Smad3 is involved in promoting tumor progression. Therefore, it is possible that Smad3 is deregulated in cancers, by interactions with an inactivating protein or epigenetic or post-translational modifications, rather than by mutation.

Since alterations in the TGF-β signaling pathway play an important role in promoting tumorigenesis and cancer progression, there is

interest in therapeutic targeting of the TGF-β signaling pathway. Selective disruption of Smad protein–protein interactions is a potential target for therapeutics. Peptide aptamers that interact specifically with Smad proteins have been developed that inhibit TGF-β responses [Cui et al., 2005; Zhao and Hoffmann, 2006]. These aptamers are 'proteins that contain a conformationally constrained peptide region of variable sequence displayed from a scaffold' that can be used to disrupt protein-protein interactions [Geyer et al., 1999]. The first class of Smad-interacting peptide aptamers interfere with the ability of Smad2 and Smad3 to interact with p300/CBP, FoxH1, and LEF1 by introducing the Smad interaction motifs from these proteins into a scaffold protein and expressing them in HepG2 cells [Cui et al., 2005]. Expression of aptamer/scaffold protein complexes specifically inhibited Smad-mediated gene expression that was dependent on the Smad-protein interaction, whereas they do not inhibit other TGF-β responses [Cui et al., 2005]. The second class of Smad-interacting peptide aptamers block TGF-β-induced signaling and EMT by inhibiting the interaction of Smad2 and Smad3 with SARA [Zhao and Hoffmann, 2006]. This suppression of TGF-β signaling is achieved by inhibition of Smad nucleocytoplasmic shuttling and complex formation with Smad4 [Zhao and Hoffmann, 2006]. In addition to blocking Smad-mediated TGF-β responses, development of other aptamers that block Smad2 and Smad3 nuclear export or protein degradation would also be effective means to activate general Smad-mediated responses. These more general peptide aptamers may be good therapeutic strategies for tumors that are dependent on TGF-β signaling for survival, cell motility, and invasion. Although the above described peptide aptamers are not selective between Smad2 and Smad3, because the proteins that were targeted bind both Smad2 and Smad3, peptide aptamers could be developed that are specific for Smad2 versus Smad3-interacting proteins. These Smad-specific aptamers may be more effective because a specific Smad response could be blocked. For example, it may not be favorable to block Smad3 signaling in tumors that are sensitive to TGF-β, as that may suppress TGF-β-mediated growth inhibition.

Although the TGF-β signaling pathway is being targeted for therapeutics in cancer patients, the tumor suppressive roles versus the tumor-promoting roles of TGF-β are not yet clearly understood. Caution should be taken with the use of these inhibitors as blocking TGF-β signaling does not always lead to inhibition of cancer cell invasion or metastasis or reduced tumor burden. Notably, TGF-β inhibitors could promote tumorigenesis through the inhibition of TGF-β signaling in normal tissues. Rather than inhibiting general TGF-β responses, inhibition of specific proteins that mediate certain TGF-β responses could provide a better strategy for therapeutic development. However, the specific TGF-β responses that are mediated by Smad2 or Smad3 are not completely understood. It is not clear if Smad2 or Smad3 or both proteins are responsible for TGF-β-induced EMT, cell motility, and invasion. As a result, determining how Smad2 versus Smad3 regulates the TGF-β signaling pathway may enable us to develop better strategies for cancer therapies.

SUMMARY AND CONCLUSIONS

The identification of the Smad signaling pathway was a major breakthrough in understanding the function of TGF-β. While a great deal of investigation has focused on the biochemical properties of Smad2 and Smad3 proteins, the signals that are regulated by Smad2 versus Smad3 are still unclear. The Smad2 and Smad3 knockout mouse phenotypes and the data comparing activation of TGF-β target genes in Smad2 and Smad3 null MEFs and in epithelial cells when Smad2 and Smad3 is silenced, suggests that Smad2 and Smad3 have distinct roles, in addition to overlapping roles in TGF-β signaling. Furthermore, the observation that TGF-β-induces growth inhibition in Smad3 null mammary gland epithelial cells, whereas the Smad3 null MEFs are resistant, suggest that the role of Smad2 and Smad3 in fibroblasts may be different than their role in epithelial cells [Yang et al., 2002]. Currently, there is not a clear understanding of the signals that result in one TGF-β response versus another and why there is selection of one response over another.

Interestingly, evidence suggests that Smad3 may have a more important role than Smad2 in TGF-β-mediated cell cycle arrest in epithelial cells [Kretschmer et al., 2003; Kim et al., 2005]. Reports by Xu et al. [2001] and Graham et al.

[1994] suggest that loss of Smad3 may largely be responsible for the non-responsiveness of some cells to TGF-β [Graham et al., 1994; Xu et al., 2001]. The proliferation, migration, and invasion of normal extravillous trophoblast cells are under the control of TGF-β. However, premalignant and malignant trophoblast cells, that have lost the Smad3 protein but retain functional Smad2, are resistant to the anti-proliferative and anti-invasive effect of TGF-β. Although evidence points to the importance of Smad3 in TGF-β-mediated inhibition of cell proliferation, it is unknown if Smad3 also has a more significant role in mediating other TGF-β responses, such as EMT. Conflicting evidence supports an active role of Smad2 and Smad3 in mediating TGF-β-induced EMT [Bhowmick et al., 2001; Valcourt et al., 2005; Zhao and Hoffmann, 2006]. It has been reported that TGF-β can induce cell cycle arrest and EMT in the same cell lines, even though both functions play contrasting roles in tumorigenesis [Brown et al., 2004]. It is a possibility that the availability of other factors such as transcription factors, co-repressors, and co-activators modulate which response is mediated by Smad3.

It is clear that Smad2 and Smad3 are differentially regulated. The regulation of Smad2 and Smad3 is complex and can occur at the level of the TGF-β receptors, nuclear import and export, protein turnover, and/or at the transcriptional level. Only importin-β and ROC1-SCF^{Fbw1a} show clear selectivity for Smad3 over Smad2 in regulating Smad3 nuclear import and ubiquitination and degradation, respectively [Xiao et al., 2000b; Fukuchi et al., 2001; Kurisaki et al., 2001]. A mechanism has not been identified that selects for Smad2 versus Smad3 nuclear export. As a result, it is likely that Smad2 versus Smad3 responses are selected by differential phosphorylation at the TGF-β receptor complex and/or through specific protein–protein interactions in the nucleus. Specifically, the adaptor protein ELF positively regulates the response of Smad3 whereas TLP activates Smad2-dependent responses [Felici et al., 2003; Tang et al., 2003]. In addition, Smad3 interacts with a number of proteins that specifically regulate Smad3-mediated transcription of TGF-β target genes.

Further studies are needed to identify the different roles and mechanism(s) of selectivity of Smad2 and Smad3 in TGF-β responses. Experiments in different cell types deficient for Smad2 or Smad3 will elucidate the roles of Smad2 and Smad3 in TGF-β signaling. As mentioned previously, TGF-β has multiple effects in different cell types and, therefore, Smad2 and Smad3 may have unique roles in those cell types. One approach to further understand the selectivity is to identify proteins that uniquely interact with Smad2 or Smad3. Previous reports have identified novel Smad2 and/or Smad3 protein–protein interactions with techniques including yeast two-hybrid assays [Akiyoshi et al., 1999; Verschueren et al., 1999; Wotton et al., 1999; Lin et al., 2000; Chen et al., 2002b; Ellis et al., 2003; Warner et al., 2003a,b, 2004; Colland et al., 2004; Wicks et al., 2005; Richard et al., 2006], luminescence-based mammalian interactome mapping (LUMIER) [Barrios-Rodiles et al., 2005], and immunoprecipitation of endogenous [Luo et al., 2006] and epitope-tagged Smad proteins [Stroschein et al., 1999; Knuesel et al., 2003; Grimsby et al., 2004] combined with mass spectrometry. These studies identified proteins that play important roles in Smad-mediated TGF-β signaling such as c-SKI, SMURF2, SNON. UCH37, SIP1, and TGIF. However, few of these studies aimed at identifying unique Smad2 or Smad3 interacting proteins in side-by-side experiments. By understanding the roles and selective activity of Smad2 and Smad3 we may be able to further comprehend the diverse roles of TGF-β in carcinogenesis and other disease processes.

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