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Smad3 is key to TGF-β-mediated epithelial-to-mesenchymal transition, fibrosis, tumor suppression and metastasis

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Abstract

Smads2 and 3 transduce signals of TGF-β from the cell surface to the nucleus. We used mice with a targeted deletion of Smad3 to study the specific contributions of this signaling pathway to pathogenic effects of TGF-β. Focusing on models involving epithelial-to-mesenchymal transition (EMT), including injury to the lens and retina of the eye and to the kidney, we have found that loss of Smad3 blocks EMT and attenuates development of fibrotic sequelae. Smad3 also plays a critical role in both the tumor suppressor and pro-metastatic effects of TGF-β in carcinogenesis. These observations suggest that development of small molecule inhibitors of Smad3 might have clinical application in treatment of fibrotic diseases as well as late stage cancers.

Keywords: Smad3; TGF-β; EMT; Fibrosis; Metastasis

1. Introduction

Elucidation of the signaling pathways downstream of TGF-β in the late 1990s has now enabled identification of specific pathways involved in mediating particular endpoints of TGF-β action both in vitro and in models of disease in vivo [1]. Given the myriad of effects of TGF-β on nearly every cell type and tissue, development of pathway-selective inhibitors for treatment of disease might ultimately lead to more specific treatments with reduced side effects (Fig. 1).

TGF-β signals through a multiplicity of pathways including the predominant Smad signaling pathway as well as mitogen-activated protein kinase pathways (MAPK; including JNK, ERK, and p38), the phosphatidylinositol-3 kinase (PI-3) pathway, and in certain cells, signaling involving PP2A phosphatase [1]. In the Smad pathway, Smads2 and 3 are activated by phosphorylation of a C-terminal phospho-serine motif by the TGF-β type I receptor (TβRI) kinase. After partnering with the common mediator Smad4, these activated Smads translocate to the nucleus where they regulate transcription of TGF-β target genes. While certain gene targets of TGF-β, such as fibronectin, appear to be activated independent of the Smad pathway [2], cDNA microarray studies suggest that the Smad pathway is generally required [3], whereas the other pathways modulate the response. For example, studies using mouse embryo fibroblasts with a targeted deletion of either Smad2 or Smad3 showed Smad3 to be an essential mediator of TGF-β responses, directly activating genes encoding regulators of transcription and signal transducers through Smad3/Smad4 (Smad3/4) DNA-binding motif repeats that are characteristic for immediate-early target genes of TGF-β but absent in intermediate target genes [3]. Interestingly, while Smad2 was not critical to the majority of the gene responses, its loss resulted in hyper-responsiveness of both immediate-early and intermediate genes to TGF-β/Smad3, suggesting that these two mediators

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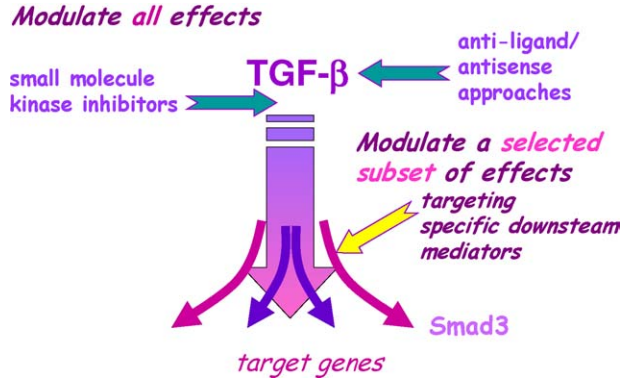

Fig. 1. Approaches to modulating the TGF-β signal transduction pathways in disease. A variety of approaches are now under development by Biotech/Pharma to modulate either the activity of the ligand or the receptor, approaches which affect all of the downstream TGF-β signaling pathways. We are proposing that pathways-selective inhibitors might have a higher degree of specificity and lesser toxicity.

play distinct roles. These differences are supported by the activity of Smads2 and 3 on the promoters of certain developmentally relevant genes such as *goosecoid*, where Smad2 activates the promoter in complex with the forkhead transcription factor, FoxH1, while Smad3 inhibits the promoter activity [4]. In contrast, inactivation of ERK/MAPK broadened the spectrum of TGF-β responsive genes. These results suggest a previously uncharacterized hierarchical model of gene regulation in which TGF-β causes direct activation by Smad3 of cascades of regulators of transcription and signaling that are transmodulated by Smad2 and/or ERK or other MAPK pathways [3].

These striking differences in Smad2/3 signaling are all the more remarkable given that they are thought to result from a gene duplication in chordates of the single Smad2/3 gene found in flies and worms [5,6]. This duplication resulted in an important basis of the difference of these two Smad proteins in terms of their ability to bind DNA directly [7]. Exon 3 of Smad2, encoding a 30 amino acid insert, is not found in Smad3 and precludes the DNA-binding of Smad2. However, a naturally occurring alternatively spliced form of Smad2 lacking exon 3 can bind interchangeably with Smad3 to the so called "Smad-binding element" (SBE) in DNA [7]. Differences become apparent following targeted deletion in mice, where deletion of Smad2 is embryonic lethal with early effects on patterning [8–10], while deletion of Smad3 surprisingly results in a viable mouse with no significant developmental lesions [11–13]. Despite these differences, possible redundant or overlapping roles of Smads2 and 3 are suggested by the recent demonstration that the lethality of embryos with a targeted deletion in Smad2 can be rescued by expression of either Smad3 or the exon 3-deleted short form of Smad2 inserted into the Smad2 locus [14], demonstrating that full length Smad2 is dispensable and that either Smad3 or the DNA-binding short form of Smad2 can support all of the developmental roles of Smad2 in activin/nodal/myostatin and TGF-β signaling pathways.

Despite the fact that the identical region of Smad2/3 (L3 loop) binds to the L45 region of the type I receptor [15,16], data suggest that the binding of these two Smad proteins in cells is not competitive. In breast cancer cell lines, we have shown that as much as 30-fold overexpression of Smad3 does not affect the phosphorylation of endogenous Smad2 and vice versa [17]. And in mouse fibroblasts with a targeted deletion of either Smad2 or Smad3, the remaining endogenous Smad is phosphorylated to a similar extent as in the wild-type cells. These results suggest either that there is a high capacity for Smad phosphorylation in these cells not exceeded by the overexpression of one Smad protein, or that Smads2 and 3 do not compete for phosphorylation by the type I receptor kinase and that the two pathways are independent and distinct [18].

2. Role of Smad3 in radiation fibrosis

Given the predominant role of Smad3 in controlling the gene targets of TGF-β in fibroblasts [3] and other reports showing that Smad3 controls synthesis of many extracellular matrix proteins, including collagens 1, 5, and 6 [19], we hypothesized that the TGF-β/Smad3 pathway might play a central role in specific pathologic fibroses in which TGF-β had previously been implicated. For example, TGF-β has been implicated in radiation fibrosis, with clinical data showing enhanced expression of TGF-β years after the initial exposure [20]. These data showing prolonged expression of TGF-β1 are supported by studies of effects of cutaneous irradiation in both pigs and mice [21,22]. Typically, radiation damage activates dermal fibroblasts, inducing expression of proteins similar to those involved in wound contraction, including alpha-smooth muscle actin (α-SMA) and collagens. Based on this, we investigated the effects of germline deletion of Smad3 on radiation fibrosis in mice to assess the contribution of this selective pathway. The results show striking protection against cutaneous injury induced by ionizing radiation in Smad3 null mice with reduced epidermal acanthosis, reduced influx of neutrophils and mast cells into the site of irradiation, reduced accumulation of myofibroblasts, and reduced accumulation of extracellular matrix compared to littermate controls at 6 weeks post-irradiation [23]. Quantitation of scarring by measuring the organization of dermal collagen fibrils also showed an improved outcome in the Smad3 null mice [24]. Importantly, the expression of TGF-β1 itself, which likely drives the fibrotic process, was also substantially reduced in Smad3 null mice, probably attenuating both the contribution of the pathogenic autocrine TGF-β-loop and the TGF-β-dependent activation of extracellular matrix protein synthesis [23]. Based on these data, we concluded that signaling through the Smad3 pathway plays a prominent role in fibrotic responses attributed to TGF-β in response to injury.

Examination of the inflammatory cell profile of Smad3 WT and null skin at 6–8 h after irradiation unexpectedly

showed a transient 5–7-fold greater influx of neutrophils into irradiated skin of Smad3 null mice as compared to WT (K. Flanders, unpublished). Preliminary results using bone marrow transplantation and skin grafting between WT and KO mice suggest that the enhanced neutrophil infiltration to irradiated KO skin results from differences in signals emanating from the irradiated KO skin compared to the irradiated WT skin, rather than from an intrinsic difference between Smad3 WT and null neutrophils (K. Flanders, unpublished). These differences are being addressed by cDNA microarray analysis of differences in gene expression patterns between irradiated WT and KO skin at short times (<24 h) post-irradiation.

3. Role of Smad3 in epithelial-to-mesenchymal transition

Certain cells have an inherent plasticity enabling them to undergo phenotypic and morphologic changes in response to signals from the environment. One example of this is the ability of epithelial cells to change their morphology and their transcriptional program to resemble that of a mesenchymal cell (epithelial-to-mesenchymal transition, EMT) [25,26]. EMT is important in development as well as in the adult in wound healing, fibrosis, and invasion and metastasis of tumor cells. Several developmental processes such as EMT of cardiac endothelial cells in formation of the endocardial cushions of the atrioventricular canal and EMT of medial edge epithelial cells in fusion of the palatal shelves, in which TGF-β had been shown to be involved, appear to be unimpaired in the Smad3 null mouse [11], suggesting that other pathways, including Smad2 might be involved [27]. Because of the known involvement of TGF-β in wound healing, fibrosis, and metastasis, processes involving EMT, we asked whether the EMT involved in these processes would also be independent of Smad3.

It is increasingly appreciated in many tissues that fibrosis can emanate from an injury to epithelial cells that then are induced to transdifferentiate into cells with a mesenchymal myofibroblastic phenotype characterized by expression of α-SMA [25]. Injury to the lens and retina of the eye are known to result in EMT of the lens epithelial cells and retinal pigment epithelial cells, respectively, with undesirable fibrotic sequelae [28,29]. While all three isoforms of TGF-β are expressed in the eye, levels of TGF-β2 are particularly high in the aqueous and vitreous and have been implicated in diseases of the eye including proliferative vitreoretinopathy (PVR), which typically leads to traction detachment of the retina and blindness [30,31]. Based on this, we investigated the role of the Smad3 pathway in two mouse models of injury-induced EMT in the eye—one involving a puncture wound to the lens mimicking the effects of cataract surgery and lens implantation [32], and the other involving partial detachment of the retina to model PVR and accumulation of matrix proteins in the vitreous [33].

EMT of lens epithelial cells following injury leads to fibrosis of the lens capsule, as can occur in the response of some patients to cataract surgery and implantation of an artificial lens [34]. The resulting deposition of extracellular matrix can lead to opacification and contraction of the capsule containing the artificial lens leading to visual impairment. Fibrosis of the anterior capsule can be modeled in the mouse by capsular injury in the lens, resulting in EMT of the lens epithelium and subsequent deposition of extracellular matrix, including lumican and collagens, without contamination of other cell types from outside the lens [35]. We anticipated that Smad3 might be involved in this process as it is activated in lens epithelial cells 12 h post-injury and its activation can be blocked by administration of a TGF-β2-neutralizing antibody in mice [34]. Moreover, we showed that EMT of primary lens epithelial cells in vitro depends on TGF-β expression [32]. Using the Smad3 null mice, we showed that these results are recapitulated in vivo and that EMT of the lens epithelium is dependent specifically on signaling through the Smad3 pathway [32]. Loss of Smad3 in mice blocks both morphologic changes of lens epithelium to a mesenchymal phenotype and expression of the EMT markers *snail*, α-SMA, lumican, and type I collagen in response to injury in vivo or to exposure to exogenous TGF-β in organ-culture. *Snail*, shown previously to be an immediate response gene target of Smad3 in fibroblasts [3], is the earliest marker differentiating effects of lens injury in Smad3 null mice and wild-type littermates. *Snail* is expressed in mouse lens epithelial cells in vitro as early as 30 min post-addition of TGF-β, and in vivo, it is detected at the edge of the capsular injury 1 day after injury, prior to expression of any other markers of EMT including lumican and type I collagen. Supporting its role as an initiating event in Smad3-dependent EMT, overexpression of *snail* in epithelial cell lines has been shown to be sufficient to induce EMT and expression of mesenchymal markers [36]. The results suggest that blocking the Smad3 pathway might be beneficial in inhibiting post-injury/-surgery capsular fibrosis.

These findings concerning a role of the Smad3 signaling pathway in ocular fibrosis also apply to a model of retinal detachment which mimics, in part, PVR, which is the most common cause of failure of reattachment surgery [33]. PVR is characterized by formation in the vitreous of a scar-like fibrous tissue containing myofibroblasts, thought to be derived from transdifferentiation of retinal pigment epithelial (RPE) cells which become activated upon being detached from the retina. Although numerous growth factors have been implicated in the disease process, including PDGF, and HGF, we have focused on TGF-β due to its correlation with disease severity [30,37,38]. The model used in this case involves making a surgical incision in the cornea, removing the crystalline lens, extruding the vitreous, and inducing a break in the peripheral retina, resulting in detachment of the retina from the underlying

pigment epithelium 1 day after surgery. One disadvantage of this model is that cellular transdifferentiation and proliferation occurs in the subretinal space and not the vitreous, but despite this, it appears to correctly model early events characteristic of PVR. Again the results of retinal detachment in Smad3 null mice compared to littermate controls were striking [33]. In wild-type mice, RPE cells proliferated under the detached retina forming pigmented fibroblast-like multilayered cells. These cells were positive for the myofibroblast marker α-SMA, and also expressed lumican and type VI collagen suggesting EMT of the RPE cells. Smad3 null mice showed none of these effects with the RPE cells remaining in a monolayer. These effects could also be modeled by excising intact eyes, injuring them in vitro, and culturing them for 48 h. Cultured primary RPE cells or an RPE cell line showed phosphorylation and nuclear translocation of Smad3 within 1 h after addition of TGF-β2 and exhibited morphologic changes to a more mesenchymal phenotype expressing α-SMA and type I collagen, again supporting a TGF-β-dependent EMT of RPE cells. Interestingly, data suggest that PDGF is also a key factor in the response of RPE cells to injury and that it is PDGF, rather than TGF-β itself, that is responsible for the proliferative response of the RPE cells [39]. Importantly, expression of PDGF is markedly suppressed in eyes of Smad3 null mice, consistent with the recent observation that induction of PDGF by TGF-β is Smad3/4-dependent [40]. These data suggest that antagonists of TGF-β or specifically Smad3, perhaps administered directly into the vitreous cavity, might be able to block the fibrogenic traction detachment of the retina not only at the level of TGF-β-mediated EMT of RPE cells with the ensuing expression of α-SMA and pathogenic accumulation of matrix proteins, but also by blocking expression of another key mediator of the disease, PDGF.

The kidney is another organ in which injury-induced EMT has been implicated in fibrogenesis. It has been suggested that the mesenchymal-to-epithelial transition of metanephric mesenchyme that occurs in embryogenesis reverses in response to injury in the form of ureteral obstruction, as would be seen in obstructive nephropathy or kidney cancer [41]. Interstitial fibroblasts that express collagen type I and the fibroblast-specific protein, FSP-1, have been shown to originate both from CD34-bone marrow cells and more prominently from EMT of resident tubular epithelial cells [42]. Again, TGF-β, among other growth factors, has been implicated in this process of tubulointerstitial fibrosis that typically is initiated by breakdown of the basement membrane and disruption of the architectural stability of the epithelial cells in contact with that membrane [43]. To directly address the role of TGF-β, and specifically the Smad3 pathway, in this process, we again employed the Smad3 null mouse, inducing injury by unilateral ureteral obstruction (UUO), considered to be a good model of obstructive nephropathy in humans [44,45] Compared to their wild-type littermates, Smad3 null mice showed reduced EMT, influx of monocytes, and accumulation of collagen, all hallmarks of the fibrotic process contributing to chronic renal failure. Primary cultures of tubular epithelial cells from Smad3 null mice showed a block in EMT in vitro and a reduction in the autoinduction of TGF-β1, consistent with the block in the generation of fibrogenic myofibroblasts from epithelial precursors in these mice in vivo. Other in vitro studies involving mechanical stretch of the primary tubular epithelial cells to mimic pathogenic effects of renal tubular distension by urine in UUO, showed that the resulting expression of α-SMA, an indicator of EMT, can be blocked in the wild-type cells by antibodies to TGF-β1 and does not occur in Smad3 null cells [45]. These data suggest that it is the injury-induced expression of TGF-β1 by the tubular epithelial cells, rather than the injury itself, that initiates EMT of the stretched cells.

Inflammatory cells also contribute to the pathogenesis of renal fibrosis, and again, the Smad3 genotype plays an important role. We had previously shown in wound healing experiments that injection of wild-type monocytes into incisional wounds made in Smad3 null mice enhanced production of matrix proteins and that chemotaxis of monocytes to TGF-β required Smad3 [46]. In UUO, wild-type monocytes showed a higher degree of influx into the wild-type renal cortex than did Smad3 null monocytes following injection of cells into the renal subcapsular space prior to ligation of the ureter [45]. No influx of either wild-type or Smad3 null monocytes was detected in similarly treated Smad3 null kidneys, consistent with the absence of TGF-β1 expression in these mice. This suggests that monocytes accelerate the EMT of obstructed kidneys and require Smad3 both for TGF-β-dependent chemotaxis and likely also for expression of TGF-β1.

Mechanistically, it is noteworthy that both in situ hybridization of the obstructed kidneys in vivo and Northern blotting of the in vitro cultured mechanically stressed tubular epithelial cells showed again that the zinc-finger transcription factor, Snail, known to be a strong repressor of E-cadherin expression, is an early marker of the EMT in vivo and that *Snail* expression is ablated in Smad3 null cells and in wild-type cells treated with antibodies to TGF-β1 [45]. Together both these data and those obtained following wounding of the lens suggest that *Snail* is a critical Smad3-dependent early response gene in TGF-β-driven EMT initiated by injury of resident epithelial cells. However, whether the homologue of *snail*, *slug*, or SIP1, another TGF-β-inducible zinc finger protein known to suppress expression of E-cadherin, might also contribute to EMT of these or other epithelial cells is not known [47,48]. Of note, in several cell lines in culture, it has been shown that the hairy/enhancer of split-related transcriptional repressor, *Hey1*, is an immediate-early gene target of Smad3 required for EMT [49]. Supporting the cell-specific induction of these immediate-early genes, *Hey1*, *Snail*, *Slug*, and *Sip1* were all co-induced by TGF-β with similar kinetics in primary kidney tubular epithelial cells, whereas in established cell

lines, induction of these genes was more restricted, with *Hey1* and *Slug* being co-induced in human keratinocyte cell line, HaCaT, and *Hey1* and *Snail* being co-induced in the human tubular epithelial cell line HK-2. These data suggest that the basic helix–loop–helix transcriptional repressor *Hey1* may play a broader role in EMT than the cell-type restricted zinc-finger transcriptional repressors *Snail*, *Slug*, and *Sip1* [49]. Importantly, none of these genes was expressed in Smad3 null epithelial cells, and EMT of wild-type cells was blocked by silencing of *Hey1*, again attesting to the Smad3-dependent role of these genes in EMT. Also noteworthy is the functional integration of Hey1-dependent activation of Jagged1/Notch signaling in EMT, and the demonstration that gene silencing of JAG1 or chemical inactivation of Notch also blocked EMT [49]. Together, these studies clearly suggest a requirement for Smad3 in injury-induced EMT-dependent fibrosis and further that putative inhibitors of Smad3 or some of its immediate-early gene targets such as *Hey1*, *Snail*, *Slug*, or *Jag1* would be especially effective in blocking the fibrotic sequelae based on both the upstream inhibition of EMT as well as inhibition of the elaboration of extracellular matrix by mesenchymal cells.

4. Role of Smad3 in carcinogenesis

The role of TGF-β in carcinogenesis is known to be very complex with well documented tumor suppressor effects on early stages of malignant transformation of cells and equally substantiated pro-metastatic effects in late stage disease, typically when the growth inhibitory response of cells to TGF-β has been lost [50]. Questions have arisen concerning whether these two seemingly contradictory functions of TGF-β, which clearly have very different gene targets and elicit different programs in the cells, are mediated through distinct signaling pathways downstream of the receptors, or whether the same signaling pathways are operative in a different contextual environment within the cell. It is well documented that cells typically exhibit reduced expression of the TGF-β receptors and enhanced secretion of the ligand during carcinogenic progression. While the original interpretation of this was that tumor cells lose their responsiveness to TGF-β, it is now known that even though certain responses to TGF-β such as growth inhibition are lost, signaling pathways are still activated and a subset of target genes expressed even in highly metastatic cells. To address this question directly in the context of a set of genetically related cell lines, we utilized a set of cells with defined carcinogenic properties all derived from MCF-10A, which originated from benign breast tissue of a woman with fibrocystic disease [51,52]. These cells have properties ranging from benign to fully malignant metastatic cells. We used these cells to modulate the Smad signaling pathway in an attempt to see whether the same manipulations would contribute to both the tumor suppressor and pro-metastatic activities of TGF-β in this system.

Based on our observations of the role of Smad3 in EMT and of the purported role of this process and of the Smad3-dependent transcription factors, Snail, Slug, and Twist in motility and invasion of metastatic cells [53–56], we chose to overexpress Smad3 as well as a dominant negative form of Smad3 in these cell lines (Fig. 2A). Surprisingly, overexpression of Smad3 in well-differentiated MCF10CA1h (CA1 h) and metastatic MCF10CA1a (CA1a) cells to as high as 30-fold over control levels had no effect on the level of phosphorylation of endogenous Smad2, suggesting that the pathways could be manipulated independently [17]. However, overexpression of the dominant negative form of Smad3 (Smad3ΔC) [57] interfered with the C-terminal phosphorylation of both endogenous Smads2 and 3 in these cells. In both cases, TGF-β-dependent signaling through the MAPK pathways was not affected. Using CA1h and CA1a cells stably altered in their Smad signaling pathway, we were able to show that in the well-differentiated CA1h cells, overexpression of Smad3 suppressed the growth of xenografted tumors injected subcutaneously into nude mice, consistent with a role in tumor suppression by TGF-β, or possibly

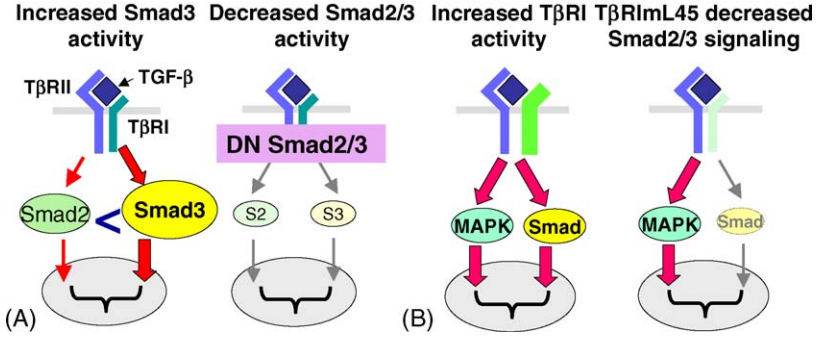

Fig. 2. Manipulations of the Smad signaling pathway (A) and TβRI (B) employed to study the effects of the Smad signaling pathway on the tumor suppressor and pro-metastatic effects of TGF-β in CA1h and CA1a breast cancer cell lines [17,59]. Both DNSmad3 with a C-terminal truncation [57], and TβRImL45, mutated in its L45 Smad-binding domain [58], block the C-terminal phosphorylation of both Smads2 and 3, while not affecting signaling through the MAPK pathways.

also activin, which could be activating that pathway in vivo. Consistent with this, interference with the signaling of the endogenous Smads by overexpression of Smad3ΔC led to enhanced growth of the xenographs. In tumors overexpressing Smad3, the more aggressive histologic sheets or cords exhibited a diffuse cytoplasmic staining for Smad3 whereas the more differentiated areas of clear cells and cribriform structures showed a prominent nuclear localization of Smad3. Moreover, the total amount of necrotic tissue and well-differentiated cribriform structures were reduced and increased, respectively, in Smad3 overexpressing tumors, consistent with a more differentiated phenotype compared to controls or tumor cells expressing Smad3ΔC. The opposite effects of enhancement of the Smad3 signaling pathway and interfering with the endogenous Smad2/3 pathways correlated with opposite effects on the degree of necrosis and the percentage of the tumor characterized by a cribriform histology [17].

Somewhat surprisingly, opposite effects of these same manipulations of the signaling pathways are seen on the metastatic CA1a cells. In this case, overexpression of Smad3 enhanced the metastasis to lung of tail-vein-injected CA1a cells, both in terms of the number of metastatic nodules and the total area of the tumors, while overexpression of Smad3ΔC markedly suppressed lung metastases compared to control [17]. Consistent with this Smad3 overexpression induced a more migratory phenotype in vitro and reduced the cell surface staining of E-cadherin, typically considered a marker of EMT and more metastatic behavior.

These results showing that the Smad2/3 signaling pathway could mediate both the tumor suppressor and pro-metastatic activities of TGF-β were confirmed using cells overexpressing either the wild-type TGF-β type I receptor (TβRI) or a receptor mutated in its L45 Smad2/3-binding loop (TβRImL45) [58] to either enhance signaling through all pathways downstream of the TGF-β receptor or to selectively suppress signaling from the endogenous Smad2/3 proteins while not interfering with MAPK signaling pathways, respectively [59] (Fig. 2B). Again, this model showed that overexpression of TβRI suppressed the growth of xenografts of CA1h cells but enhanced the number and size of lung metastases of tail-vein-injected CA1a cells. Conversely, overexpression of TβRImL45 elicited the opposite response, enhancing the growth of the xenografted CA1h cells and suppressing the metastatic activity of the CA1a cells by interfering with the Smad-mediated tumor suppressor pathway and the Smad-mediated pro-metastatic activity, respectively [59].

These results clearly argue for a difference in the context of the Smad2/3 signaling pathway in CA1h versus CA1a cells (Fig. 3). Exactly what constitutes this switch in the gene targets is still unknown. Microarray analysis of tumors from CA1a and CA1h cells shows distinct differences in a subset of gene targets (B. Tang and L.M. Wakefield, unpublished) even though each of these

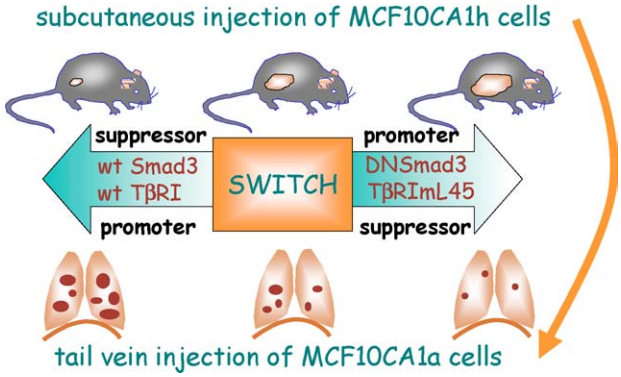

Fig. 3. The tumor suppressor and pro-metastatic activities of TGF-β are mediated through common signaling pathways dependent on the context of the tumor cell. The Smad2/3 pathway can mediate both tumor suppressor and pro-metastatic activities of TGF-β in opposite ways in xenografts of the well-differentiated CA1h cells and in lung metastases of CA1a cells. This clearly indicates a switch in the gene programming mediated through this pathway as cells progress through increasingly malignant stages of carcinogenesis.

cells originated from sequential in vivo and in vitro passage of the Ras-transformed MCF10A derivative MCF10At1k [51]. Studies from a series of pancreatic cell lines, some of which have lost their ability to be growth inhibited by TGF-β, but nonetheless can still activate Smad2/3 signaling in response to TGF-β, suggest that there is a shorter nuclear residence time of phosphorylated Smad2/3 complexes in cells which have lost the growth inhibitory response and which express lower levels of TβRI [60]. Importantly, this shorter nuclear residence time correlates directly with an altered pattern of target gene expression, with expression of certain genes such as *PAI-1*, *Smad7*, and *c-Jun*, all implicated in tumor promotion, being maintained, but expression of other cell-cycle-related gene targets such as *p21* and *p15*, involved in the growth suppressive activities of TGF-β, being either severely attenuated or absent. The authors propose that the reduced level of the TGF-β receptors commonly observed on more malignant, metastatic tumor cells could directly lead to this shorter nuclear residence time of the Smad proteins, and thereby to an altered pattern of transcription [60].

Since it is becoming increasingly appreciated that tumor stroma plays a significant role in the tumor phenotype [61,62] and since effects on stromal components of TGF-β secreted by the tumor cell have long been implicated in carcinogenesis [50], we have also been investigating differences in the CA1h and CA1a cells in co-culture with fibroblasts. We have found that the secretion of the basement membrane protein degrading MMP-9 by the fibroblasts surrounding the tumor cell islets is influenced by the degree of tumorigenicity of the tumor cell, with the CA1a cells inducing secretion of the highest amounts of this enzyme, which has been implicated in metastasis [63]. Use of specific inhibitors again implicates the Smad signaling pathway in

this process, though likely through a combination of the actions of Smads 2 and 3.

5. Potential for development of pathway-specific inhibitors

Given the clear involvement of TGF-β in wound healing, fibrotic diseases, and carcinogenesis, there is now heightened interest in developing drugs to modulate this pathway (Fig. 1) either by judicious application of the ligand itself [64], antisense or antibody approaches to interfering with the ligand, or small molecule inhibitors of the TβRI kinase, which ablate all signaling pathways downstream of the receptors [65]. Other approaches involved the use of naturally occurring inhibitors such as halofuginone, originally used as an anticoccidial in poultry feed and subsequently shown to inhibit TGF-β-induced expression of collagen alpha 1(I) [66] and some of the unwanted effects of radiation fibrosis through suppression of the Smad signaling pathway [67]. Surprisingly, studies indicate that some of these approaches, especially those directed to the ligand itself, including either antibodies or soluble receptor constructs, appear to be dependent on access of these reagents to the ligand, and may be efficacious at modulating the disease process without affecting basal homeostatic effects of TGF-β [68,69]. In contrast, small molecule inhibitors of the receptor kinase are likely to block all effects of TGF-β and may have more unwanted side effects. What we are proposing, based on the critical role of Smad3 in many of the pathogenic effects of TGF-β in both fibrosis (especially fibrosis involving EMT), and metastatic disease (Fig. 4), is that future development be directed at pathway-specific inhibitors to increase specificity and decrease unwanted side effects (Fig. 1). The concept of this approach is being explored using Smad-interacting peptide aptamers based on Smad-interacting sequences in proteins such as FoxH1, Lef1 and CBP [70]. Initial interactions show very selective interference with expression of TGF-β gene targets and hold promise for even more selective approaches in the future.

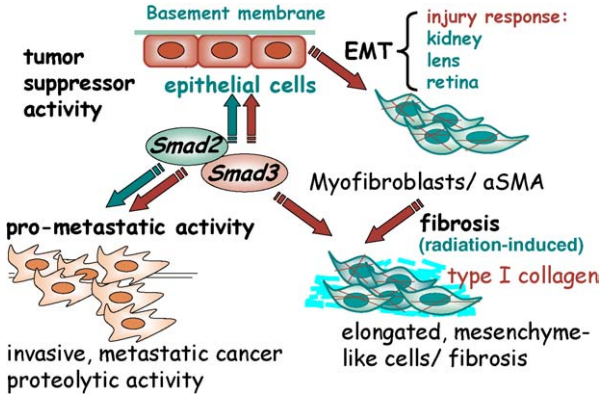

Fig. 4. Smad3 is required for effects of TGF-β in injury-induced EMT, fibrosis, as well as in tumor suppression and metastasis.

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