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PROSPECTS

Pro-Metastasis Function of TGFβ Mediated by the Smad Pathway

Yibin Kang*

Department of Molecular Biology, Princeton University, Princeton, New Jersey

Abstract The transforming growth factor beta (TGFβ) signaling pathway plays a vital role in the development and homeostasis of normal tissues. Abnormal function of this pathway contributes to the initiation and progression of cancer. Smad proteins are key signal transducers of the TGFβ pathway and are essential for the growth suppression function of TGFβ. Smads are bona fide tumor suppressors whose mutation, deletion, and silencing are associated with many types of human cancer. However, the involvement and functional mechanism of Smad proteins in cancer metastasis are poorly defined. Recent studies using genetically modified cancer cells and mouse tumor models have provided concrete evidence for a Smad-dependent mechanism for metastasis promotion by TGFβ. Understanding the dual roles of Smad proteins in tumor initiation and progression has important implications for cancer therapeutics. J. Cell. Biochem. 98: 1380–1390, 2006. © 2006 Wiley-Liss, Inc.

Key words: TGFβ; Smad; mouse model; tumor progression; metastasis

Genes involved in oncogenic pathways are generally classified as either tumor suppressors or oncogenes, depending on their function in regulating cell growth, differentiation, and death. This arbitrary demarcation between peacekeepers and evildoers, however, is often ambiguous. A recurring theme in molecular oncology is that a tumor suppressor can become an oncogene under certain circumstances, and vice versa. Transforming growth factor beta (TGFβ) and its downstream signal transducers are well-documented molecules with such a paradoxical character. TGFβ family cytokines, including TGFβ1, β2, and β3, are members of a large superfamily of pleiotropic growth factors that includes the activins and bone morphogenetic proteins (BMPs) [Roberts and Sporn, 1990; Massagué, 1998; Shi and Massagué, 2003]. TGFβ family cytokines regulate complex physiological processes such as cell proliferation, differentiation, adhesion, matrix production, motility, and apoptosis. TGFβ was initially discovered as one component of a secreted activity from tumor cells that could produce a transformed phenotype in normal fibroblast. Subsequently, this activity was identified as TGFα, an EGF receptor ligand and a strong growth-stimulating factor, and TGFβ, a potent growth inhibitor of epithelial cells, hematopoietic cells, neuronal cells, endothelial cells, and many other cell types [Roberts and Sporn, 1990]. With the identification of inactivating mutations within the components of the TGFβ pathway in cancers, it became clear that TGFβ is indeed a tumor suppressor pathway for many different types of cancer. However, late stage human carcinomas often become resistant to TGFβ growth inhibition and, in addition, secrete elevated levels of TGFβ [Reiss and Barcellos-Hoff, 1997; Derynck et al., 2001]. Genetic manipulation of the TGFβ pathway in tumor cell lines and experimental animal models validated the metastasis-promoting function of TGFβ in late stage cancer progression [Letterio, 2005]. Although TGFβ receptors and Smad transcription factors are the best-characterized TGFβ signal transducers, TGFβ also signals through several Smad-independent kinase pathways [Derynck and Zhang, 2003]. It is not clear whether Smad proteins, which are typical tumor suppressors, can also mediate the pro-metastatic function of TGFβ. Understanding the stage specific duality of TGFβ and Smad

*Correspondence to: Yibin Kang, PhD, Department of Molecular Biology, Washington Road, LTL 255, Princeton University, Princeton, NJ 08544.
E-mail: ykang@molbio.princeton.edu

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function and the molecular mechanism underlying the role reversal of TGFβ in tumor progression has become a critical issue in molecular oncology.

THE ESSENTIALS OF THE TGFβ PATHWAY

Our current understanding of the biochemical, structural, and functional properties of the TGFβ-Smad pathway has been discussed in great detail by several excellent recent reviews [Attisano and Wrana, 2002; Shi and Massagué, 2003; Massagué et al., 2005]. Here I will briefly summarize the essentials of the TGFβ-Smad pathway as an introduction to the discussion of its dual role in cancer. TGFβ proteins are produced as latent dimers with inhibitory prosegments. Several tissues, such as bone matrix, serve as a reservoir of latent TGFβ, which can be readily activated by a number of proteases, such as plasmin, MMP2, and MMP9. As these proteases are frequently overexpressed by tumor cells, active TGFβ is often found to be present at high levels in extracellular matrix (ECM) at the active invasion front of tumor cells [Reiss and Barcellos-Hoff, 1997]. Activated TGFβ binds to a heterodimeric cell surface receptor complex consisting of a pair of transmembrane serine/threonine kinases, TGFβ type I (TβRI) and type II (TβRII) receptors. After ligand binding to TGFβ receptors, TβRII phosphorylates the GS domain upstream of the kinase domain in TβRI, resulting in the activation of TβRI kinase activity. The Smad proteins are the only known signal transducers that consistently transmit the TGFβ signal from the cell membrane to the nucleus. Receptor-regulated Smads (R-Smads) interact transiently with specific activated type I receptors and become phosphorylated at the C-terminus. Smad2 and Smad3 are specific mediators of TGFβ/Activin/Nodal pathways, whereas Smad1, Smad5, and Smad8 are involved in the signaling of other members of the TGFβ/BMP superfamily of cytokines, such as BMP. Smad4 is a common mediator (Co-Smad) of TGFβ and BMP signaling. It forms hetero-oligomers with the phosphorylated R-Smads and the complex accumulates in the nucleus to effect transcriptional changes. Smad6 and Smad7 are inhibitory Smads (I-Smads) that compete with R-Smad for binding to TβRI and prevent the phosphorylation and activation of R-Smads by TβRI [Shi and Massagué, 2003]. Smad3 and Smad4 have low intrinsic DNA binding affinity to a simple consensus binding sequence AGAC (or the anti-parallel sequence GTCT). The target gene specificity is determined by composite DNA motifs consisting of the Smad binding sites and one or more binding sites for other transcription factors. A growing list of transcriptional factors, co-activators and co-repressors are being identified to be Smad transcriptional partners [Massagué et al., 2005]. Smad proteins often interact with these factors through a "hydrophobic corridor" in the MH2 (Mad Homology 2) domain [Massagué et al., 2005]. The transcriptomic output of TGFβ signal transduction is determined by the receptor signaling strength, the cellular context of Smad cofactors, the composition and epigenetic status of Smad target sites and the activity of signaling pathways that can modify or influence the components of the TGFβ-Smad pathway. Hundreds of genes can be activated or repressed by TGFβ in a given cell type, although only a portion of the gene responses are ubiquitous in all cells, with the rest being cell type-specific gene responses. In addition to Smads, other signaling pathways have also been implicated in TGFβ actions downstream from the TGFβ receptors [Derynck and Zhang, 2003]. These include the extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK), phosphatidylinositol-3 kinase (PI3K), TGFβ-activated kinase 1 (TAK1), protein phosphatase 2A (PP2A), and Rho GTPases. To what extent TGFβ can activate these Smad-independent pathways depends on the cell type and physiological condition of the cells. In addition to targeting downstream genes directly, these pathways also indirectly influence the outcome of TGFβ signal transduction by modifying the activity and strength of the Smad pathway [Derynck and Zhang, 2003].

SMAD PROTEINS AS TUMOR SUPPRESSORS

The most profound and well-characterized physiological consequence of TGFβ signaling in epithelial cells is growth inhibition (Fig. 1). Transcriptional profiling experiments on different types of epithelial cells reveal a core TGFβ cytostatic gene response program that includes the downregulation of c-Myc and Id family of transcription factors (Id1, Id2, and Id3) and activation of $p15^{INK4b}$ and $p21^{CIP}$ cyclin-dependent kinase inhibitors [Kang et al., 2003a].

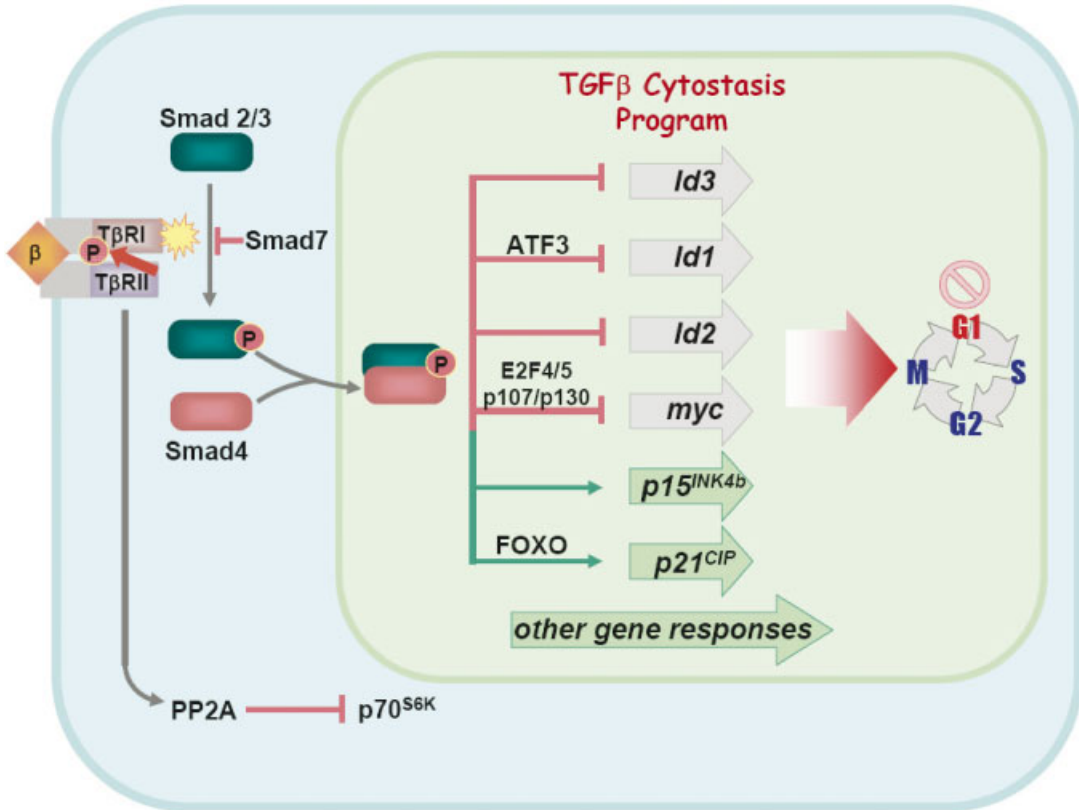

Fig. 1. Transforming growth factor beta (TGFβ) inhibit cell cycle progression in normal epithelial cells. TGFβ controls homeostasis and inhibits tumor formation through transcriptional regulation of genes that are important for cell cycle progression. Cell cycle-related genes that are commonly regulated by the TGFβ-Smad pathway in various different types of normal epithelial cells are listed. See text for detailed descriptions. Under particular circumstance, TGFβ may also inhibit cell cycle progression through Smad-independent pathways, for example, through activation of protein phosphatase 2A (PP2A) and inhibition of p70 S6 kinase [Petritsch et al., 2000]. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Smad proteins interact with a number of different transcriptional cofactors to bring about the cytostatic gene responses to TGFβ. Thus, Smad3 and Smad4 form a transcriptional repression complex with E2F4, E2F5, and pocket proteins p107 and p130 on a composite Smad-E2F binding site located at the *c-Myc* promoter [Chen et al., 2002]. Inhibition of the *Id1* gene is mediated by the interaction of Smad3 with ATF3, a transcriptional repressor that is induced by TGFβ [Kang et al., 2003a]. TGFβ represses *Id2* expression by increasing the transcription of Myc antagonistic repressors Mad2 and Mad4, resulting in the replacement of Myc–Max activation complexes with Mad–Max repression complexes on the *Id2* promoter [Siegel et al., 2003b]. Activation of *p15* and *p21* expression is mediated by the Smad–FoxO complex on the *p15* promoter [Seoane et al., 2004] and an unknown Smad complex on the *p21* promoter. Reduction of Myc protein level by TGFβ signaling additionally alleviates the transcriptional repression of *p15* and *p21* by the Myc–Miz repression complex on both genes [Seoane et al., 2001]. Through the concerted action of inhibiting growth-promoting genes (*c-myc* and *Id* family genes) and activating CDK inhibitors, TGFβ can potently arrest cell cycle at the G1 checkpoint.

Consistent with its role in growth inhibition, TGFβ has been shown to function as a tumor suppressor pathway in clinical studies and mouse model experiments. Reduction or loss of TGFβ receptors, Smad2 or Smad4 occurs frequently in pancreatic, colorectal, gastric, breast and many other types of cancers [Massagué et al., 2000; Derynck et al., 2001]. Smad4, initially identified as DPC4 (deleted in pancreatic carcinoma locus 4), is deleted or mutated in about 50% of pancreatic cancers [Hahn et al., 1996], 30% of metastatic colon tumors [Miyaki et al., 1999], and in a smaller fraction of other

carcinomas. In addition, a Smad4 germline mutation causes familial juvenile polyposis, an autosomal dominant disease characterized by a predisposition to hamartomatous polyps and gastrointestinal cancer [Howe et al., 1998]. Smad2 mutation was found in a small proportion of colorectal cancers [Eppert et al., 1996; Uchida et al., 1996]. Although point mutations have not been described for Smad3 from human cancer, Smad3 has recently been shown to be an important tumor suppressor in pediatric T-cell acute lymphoblastic leukemia (ALL) and gastric cancer [Han et al., 2004; Wolfraim et al., 2004]. Leukemic cells from children with T-cell ALL lack Smad3 protein although they contain normal levels of *Smad3* mRNA [Wolfraim et al., 2004]. Over one third of human gastric cancer tissues showed low to undetectable levels of Smad3 and the same proportion of gastric cancer cell lines is deficient in Smad3. Introduction of Smad3 into human gastric cancer cells that did not express Smad3 restored the growth inhibition response to TGFβ in vitro and delayed tumorigenicity in vivo [Han et al., 2004]. Thus, unlike a typical tumor suppressor, which is often inactivated by loss of heterozygosity, Smad3 appears to be silenced by epigenetic mechanisms.

Results from transgenic animal experiments have validated the tumor-suppressive role of the TGFβ-Smad pathway in mammary gland, skin, colon and other organs. Overexpression of TGFβ1 in transgenic mice inhibits the formation of carcinogen-induced mammary and skin tumors [Pierce et al., 1995; Cui et al., 1996]. On the other hand, mice heterozygous for the *Tgfb1* gene have a reduced level of TGFβ1 protein and increased risk of developing carcinogen-induced tumors [Tang et al., 1998]. Similarly, mice with targeted disruption of either *Tgfb1* or *Smad3* are prone to develop colon cancer [Zhu et al., 1998; Engle et al., 1999; Xu et al., 2000], and mice carrying an inactivated allele of *Smad4* develop intestinal polyps that progress to carcinoma [Xu et al., 2000]. Attenuation of autocrine TGFβ signaling by expression of a dominant-negative TβRII results in enhanced propensity for carcinogen-induced lung, mammary, and skin tumors [Bottinger et al., 1997; Go et al., 1999], and oncogene-induced mammary carcinomas [Gorska et al., 2003; Siegel et al., 2003a]. Disruption of Smad signaling by tissue-specific expression of Smad7 promotes the formation of pre-malignant ductal lesions in the pancreas in transgenic animals [Kuang et al., 2006].

THE PRO-METASTATIC FUNCTION OF THE TGFβ PATHWAY

Despite the high incidence of TGFβ receptor and Smad mutations in colorectal and pancreatic cancers, cancers arising from other organs often maintain an intact TGFβ pathway but with a diminished response to TGFβ growth inhibition [Massagué et al., 2000; Chen et al., 2001; Derynck et al., 2001]. This reduction in TGFβ responsiveness is often accompanied by increased secretion of TGFβ isoforms by tumor cells during the progression of many types of cancers, including breast, colon, prostate, bladder, pancreatic, and gastric cancers, and melanoma [Reiss and Barcellos-Hoff, 1997; Massagué et al., 2000; Derynck et al., 2001]. Furthermore, increased TGFβ production is often associated with poor clinical outcome, such as higher risk of metastasis and shorter survival time, which suggests that the excessive amount of TGFβ may actively promote malignant progression.

Animal tumor model experiments have also provided supporting evidence for an active role of TGFβ in promoting malignancy of late stage tumors. Carcinogen-induced tumors that develop in TGFβ1 haploid mice often maintain the wild-type TGFβ1 allele and these tumors in fact produce higher level of TGFβ1 than tumors from the wild-type mice [Tang et al., 1998]. Mice overexpressing active TGFβ in keratinocytes develop fewer benign papillomas compared with controls. However, once tumors develop, the transgenic tumors rapidly acquire a spindle cell phenotype, overexpress TGFβ, and metastasize [Cui et al., 1996]. Overexpression of active TGFβ1 or a constitutively active form of TβRI in the mammary gland of transgenic mice accelerated metastases derived from neu-induced primary mammary tumors [Muraoka et al., 2003; Siegel et al., 2003a]. These observations have lead to the speculations that during cancer progression, TGFβ may reverse its role from an inhibitor of tumor growth to a tumor promoter [Reiss and Barcellos-Hoff, 1997; Massagué et al., 2000; Derynck et al., 2001]. Although complete or partial loss of TGFβ signals is permissive for early stages of tumor development, active TGFβ signaling (with selective loss of growth inhibitory response) may be

advantageous for the progression and metastasis of cancer.

Clinical and experimental metastasis studies, however, reveal controversial results when the prognosis is correlated with the expression status of TGFβ receptor in cancer cells. For instance, loss of TβRII expression correlates with poor prognosis in breast, esophageal, cancer, and renal carcinoma [Gobbi et al., 1999, 2000; Fukai et al., 2003; Miyajima et al., 2003] but correlates with a better survival rate in colon cancer [Watanabe et al., 2001] and gastric cancer [Miyajima et al., 2003]. In animal experiments, abrogation of TGFβ signaling in mouse mammary and colon cancer cells by the expression of a dominant-negative TβRII was shown to inhibit their in vivo growth and metastasis [Oft et al., 1998]. Administration of a TGFβ neutralizing antibody inhibited the tumorigenicity of the MDA-MB-231 cells in nude mice [Arteaga et al., 1993]. Dominant-negative TβRII expression in human breast carcinoma MDA-MB-231 cell was also shown to inhibit its bone metastatic potential by blocking TGFβ-induced tumor production of parathyroid hormone-related protein (PTHrP), an osteoclast-activating hormone. The constitutively active form of TβRI can restore the bone metastasis ability of these cells [Yin et al., 1999]. Similarly, soluble TβRIII (betaglycan) or TβRII were shown to inhibit tumorigenesis and metastasis of the breast cancer in animal models [Muraoka et al., 2002; Yang et al., 2002]. In the MCF10A series of human mammary epithelial cell lines that are genetically related and represent different stages of tumor progression, blockade of TGFβ signaling by dominant-negative TβRII was shown to promote tumorigenicity of a low grade pre-malignant cell, but inhibited metastasis of a high-grade tumorigenic cell [Tang et al., 2003; Tian et al., 2003, 2004]. Decreased breast cancer metastasis was also seen in a model expressing a dominant-negative TβRII (ΔβRII) in human breast-derived cell lines [Tang et al., 2003]. In contrast, increased skin cancer metastasis and prostate cancer metastasis were observed in the respective ΔβRII transgenic models [Go et al., 1999; Tu et al., 2003]. Loss of TβRII in the context of polyomavirus middle T antigen expression in mammary tumor results in a shortened tumor latency and an increased formation of pulmonary metastases [Forrester et al., 2005]. These often contradictory observations highlight the complex nature of the functions of TGFβ in metastasis, which could be stage and/or tissue specific, or may be dependent on the context of other oncogenic mutations.

Several functional mechanisms have been described to explain the metastasis-promoting function of TGFβ (Fig. 2). TGFβ alters the composition of ECM by activating the production of ECM components such as fibronectin and laminin, or ECM-modifying enzymes such as matrix metalloproteases [Derynck et al., 2001]. TGFβ can enhance angiogenesis by activating the expression of pro-angiogenic factors such as FGF and VEGF [Yang and Moses, 1990]. TGFβ can also suppress the immune surveillance by inhibiting the proliferation, activation, and differentiation of lymphocytes and by repressing the expression of cytolytic gene products in cytotoxic T lymphocytes (CTLs) [Arteaga et al., 1993; Letterio and Roberts, 1998; Thomas and Massagué, 2005]. Additionally, TGFβ can promote cancer progression through cell-autonomous mechanisms, such as promoting epithelial mesenchymal transition (EMT), a process that is associated with enhanced migration and invasion of tumor cells [Miettinen et al., 1994; Oft et al., 1996]. The functional importance of TGFβ-dependent EMT was validated by introduction of dominant-negative TβRII into a variety of metastatic carcinoma cells, which prevents conversion of tumor cells to a mesenchymal phenotype and inhibits motility, tumorigenicity, and metastases [Oft et al., 1998; Han et al., 2005]. Additionally, TGFβ can directly stimulate the expression of tissue-specific metastasis genes and mediate the pro-metastasis crosstalk between tumor cells and the stromal environment. Through these various autocrine or paracrine mechanisms, TGFβ may contribute to the progression and metastasis of cancer.

THE ROLE OF SMAD PROTEINS IN METASTASIS PROMOTION

In contrast to the detailed knowledge of Smads in the anti-mitotic branch of TGFβ transcription regulation network, the role of Smads in the metastasis-enhancing branch of TGFβ remains poorly defined and controversial. Although TGFβ receptors have been shown to be essential for promoting cancer metastasis, it is not clear whether Smad proteins are required

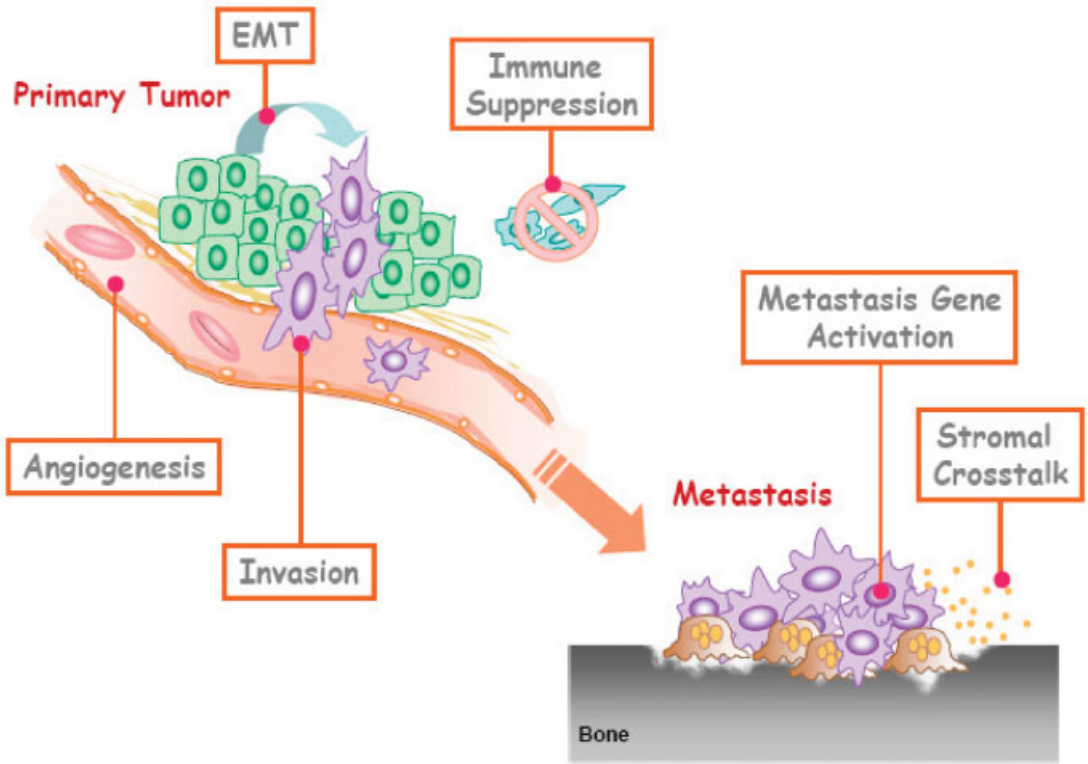

Fig. 2. The pro-metastasis function of the TGFβ pathway in late stage tumors. TGFβ promote the progression and metastasis of late stage cancer by inducing epithelial-mesenchymal transition or cancer cells, by immune suppression, by promoting angiogenesis, invasion and metastasis gene expression, and by mediating the crosstalk between tumor cells and the stromal components. Cancer cells often become refractory to TGFβ growth inhibition while maintaining the pro-metastasis responses to TGFβ. [Color figure can be viewed in the online issue which is available at www.interscience.wiley.com.]

for the pro-metastatic function of the TGFβ signaling pathway. Several reports have suggested that the involvement of TGFβ signaling in metastasis is mediated by Smad-independent pathways and that Smad proteins may still play a tumor-suppressive role in late stage cancer. For example, Iglesias et al. showed that Smad4 inhibits Ras-dependent Erk signaling activity in Ras-transformed keratinocytes. Expression of dominant-negative Smad4 in these cells results in hyperactivation of Erk signaling and malignant progression [Iglesias et al., 2000]. Expression of a dominant-negative TβRII in the MDA-MB-231 breast cancer cell line inhibits motility of these cells in vitro, which can be restored by expression of a constitutively active form of TβRI, but not by overexpression of Smad proteins [Dumont et al., 2003]. Expression of activated TβRI enhances Akt and ERK activities but not Smad2 phosphorylation, suggesting that TGFβ promotes motility through mechanisms independent of Smad signaling, possibly involving activation of the PI3/Akt and/or MAP kinase pathways [Dumont et al., 2003]. Furthermore, TGFβ-induced cell cycle arrest and migration, but not epithelial-mesenchymal transition, are abolished in HaCaT keratinocytes after silencing of Smad4 [Levy and Hill, 2005].

Abundant evidence also exists to support an active role of Smad proteins in mediating a cell-autonomous mechanism of metastasis enhancement by TGFβ. Early work by Oft et al. demonstrated that the capability of TGFβ signaling to cooperate with H-Ras to promote EMT, cell motility, and metastasis is at least in part mediated by Smads [Oft et al., 1996, 2002]. Dominant-negative Smad3 inhibits lung metastasis of breast cancer cells in animal models [Tian et al., 2003]. Two recent studies used the I-Smad, Smad7, to modulate the metastatic ability of mammary and melanoma tumors [Azuma et al., 2005; Javelaud et al., 2005]. Overexpression of Smad7 in JygMC(A) mouse mammary tumor cells lead to a dramatic decrease in metastases and increased survival

in animal experiments. The decrease in metastasis was associated with increased expression of major components of adherens and tight junctions, decreased expression of N-cadherin, and decreases in the migratory and invasive abilities of the cancer cells [Azuma et al., 2005]. Stable overexpression of Smad7 in a highly metastatic human melanoma cell line inhibited tumor cell invasiveness in vitro and malignancy in vivo, without altering their proliferation capacity [Javelaud et al., 2005]. Therefore, evidence arguing for or against a pro-metastatic role of Smad both exists. The inconclusive and sometimes contradictory reports about the role of TGFβ pathway components in metastasis may be the result of different tumor types, tumor stages, oncogenic backgrounds, experimental systems, functional assays, and pathway modulating reagents that were used in different studies.

THE SMAD PATHWAY IN BONE METASTASIS OF BREAST CANCER

Bone metastasis is a major cause of morbidity for patients with breast, prostate, lung and other types of cancers. Over 70% of late stage breast cancer patients have bone metastases that cause severe pain, pathological fractures, and hypercalcemia [Mundy, 2002]. Bone is a rich reservoir of latent TGFβ and osteolytic bone metastasis by breast cancer often leads to the activation of latent TGFβ [Kang et al., 2005], making bone metastasis an ideal model to study the involvement of TGFβ in cancer metastasis. The MDA-MB-231 breast cancer cell line has been widely used in an animal model of osteolytic (bone-degrading) metastasis. Introduction of tumor cells into the systemic arterial circulation of recipient nude mice by intracardiac injection routinely leads to multiple bone metastases in fore- and hind-limbs, spinal cord, and skull within 7–12 weeks after injection [Yin et al., 1999; Kang et al., 2003b]. The involvement of TGFβ signaling in bone metastasis was clearly demonstrated by Yin et al. [1999] using ectopic expression of mutant TGFβ receptors in the MDA-MB-231 cells. Expression of dominant-negative TβRII led to inhibition of bone metastasis, while a constitutively active form of TβRI was able to restore the bone metastatic potential of these cells. The bone metastasis-enhancing function of TGFβ was partly mediated by the increased secretion of the PTHrP, an osteoclast activator, from cancer cells through a mechanism that is dependent on both the Smad and the MAP kinase pathways [Yin et al., 1999; Kakonen et al., 2002]. Other candidate bone metastasis genes were identified by transcriptomic profiling of highly metastatic cells selected in vivo from bone lesions generated by the parental, mildly metastatic MDA-MB-231 cell line. These genes includes the bone homing receptor *CXCR4*, the osteoclast-activating cytokine *Interleukin-11* (*IL-11*), the proteolytic factor *MMP1* (*matrix metalloprotease-1*, or *collagenase-1*), the angiogenic factor *CTGF* (*connective tissue growth factor*) and others [Kang et al., 2003a, 2005]. The functional role of these genes in bone metastasis was validated by metastasis assays of cell lines engineered to overexpress these genes individually or in combination. Importantly, among these genes, *IL11* and *CTGF* are potently activated by TGFβ signaling in MDA-MB-231 cells, while *CXCR4* and *MMP1* are known to be transcriptional targets of TGFβ in other cell lines. Therefore, TGFβ present at the bone metastasis site can activate multiple metastasis genes to fuel a vicious cycle for bone destruction [Mundy, 2002; Guise et al., 2005]. It is conceivable that the list of metastasis genes that are activated by TGFβ will continue to grow as functional genomic study of cancer metastasis continues to yield novel insights about the complex network of genes and pathways that mediate metastasis.

We used retroviral expression of Smad4-silencing short hairpin RNA (shRNA) in MDA-MB-231 to investigate the role of the Smad pathway in bone metastasis. Smad4 is the only Co-Smad in human genome and is essential for Smad signal transduction. Microarray profiling of Smad4-knockdown cells suggested that the ablation of Smad4 abolished most of the TGFβ gene responses. Expression of two different shRNAs against Smad4 led to efficient reduction (over 90%) of endogenous *Smad4* transcript in the MDA-MB-231 cells. When these cells were injected into nude mice, bone metastasis was significantly reduced, although not completely eliminated. Importantly, when a shRNA-insensitive Smad4 cDNA was stably expressed in Smad4-knockdown cells, the bone metastatic efficiency of the tumor cells was restored. *IL-11* and *CTGF*, two bone metastasis genes that are transcriptional targets of TGFβ signaling, was no longer responsive to TGFβ in Smad-knockdown cells. Ectopic expression of

Smad4 restored the TGFβ responsiveness of these genes. This series of experiments clearly demonstrated the Smad-dependency for the bone metastasis-promoting function of TGFβ.

We also used reporter-based in vivo imaging to analyze the difference of TGFβ signaling activities at various target organ sites for metastasis [Kang et al., 2005]. A retrovirus carrying a thymidine kinase (TK) reporter under the control of a TGFβ-responsive promoter and another retrovirus carrying a firefly luciferase reporter under the constitutively active CMV promoter were used to co-infect breast cancer cells. Bioluminescence imaging of the luciferase allows us to track the location of metastasis and measure the relative size of the secondary tumors. Micro-PET imaging using the TK reporter produced an in vivo readout of the TGFβ signaling activity in different metastasis sites. Interestingly, the strength of TGFβ signaling was much stronger in bone than in other tissue sites such as the adrenal medulla. This result suggested that the functional importance of TGFβ-Smad pathway in metastasis may vary between different metastasis target sites. Therefore, differences in stromal environment need to be taken into account when interpreting experimental results from animal experiments and considering TGFβ pathway antagonists as anti-metastatic agents [Dumont and Arteaga, 2003].

FUTURE PERSPECTIVES

Clinical and experimental studies of cancer metastasis have begun to shed light on the involvement of Smad proteins in the metastasis-enhancing function of TGFβ. Whether Smad proteins are suitable targets for therapeutic interventions awaits more conclusive results from future studies. Many questions about the functional mechanism of Smad pathway in cancer metastasis remain unanswered. What is the involvement of other TGFβ-related, Smad4-dependent pathways, such as the BMP pathway, in metastasis? Is there any functional difference between Smad2 and Smad3 in metastasis, as there is with respect to growth inhibition? What is the role of the TGFβ-Smad pathways in the metastasis of cancer cells to target organs other than bone? What are the TGFβ target genes that contribute to different types of tissue-specific metastasis and what are the Smad cofactors that mediate the responses of these genes to TGFβ? Can we develop targeted therapeutics that specifically inhibit these responses without blocking the growth suppressive function of TGFβ in normal cells? Can we detect any molecular signature in the primary tumors or metastases that can predict the efficacy of anti-TGFβ therapy? Sophisticated genetic manipulation tools for cell lines and animal models as well as advanced in vivo imaging, genomic profiling, and computational modeling will help provide answers to these questions in the coming years.

Better understanding of the stage- and tissue-specific functions of the TGFβ-Smad pathway will benefit from experimental systems that modulate the pathway in a temporal- and spatial-specific manner. Inducible expression of TGFβ in mouse papillomas and mammary tumor has been used to demonstrate a causal role of TGFβ in metastasis progression [Weeks et al., 2001; Muraoka-Cook et al., 2004]. More strikingly, tissue-specific knockout of TβRII in fibroblasts was recently shown to promote mammary carcinoma growth and invasion through upregulation of TGFα and HGF-mediated signaling networks [Cheng et al., 2005]. Innovative designs of animal models that can precisely alter TGFβ activity in specific cell types and tumor stage will not only enrich our understanding about the functional mechanism of TGFβ in cancer progression, but also facilitate the development of TGFβ-targeted therapeutics.

Although life-long exposure to a soluble TGFβ receptor antagonist did not lead to apparent adverse side effects in animal models [Muraoka et al., 2002; Yang et al., 2002], it remains to be seen whether this is also true in clinical trials. Inhibitors that specifically target the pro-metastasis branch but not the anti-mitogenic branch of the TGFβ pathway could potentially be a better alternative than non-selective inhibitors. It is still not clear how cancer cells maintain the pro-metastatic responses to TGFβ while selectively losing the growth inhibitory response. Transcriptional activity of Smad proteins can be inhibited or altered by phosphorylation by MAP kinase pathway, CDKs and other kinases that are often hyperactive in cancer cells [Matsuura et al., 2004; Massagué et al., 2005]. It appears that the anti-mitogenic gene responses to TGFβ are more sensitive to the reduction of Smad activity, while pro-metastasis responses are more resistant to, or

even dependent on the crosstalks from other kinase pathways. Designing branch-specific inhibitors for the TGFβ-Smad pathway will rely on a better understanding of the complex network of cellular cofactor and signaling pathways that influence TGFβ signaling downstream of Smad activation.

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