TRANSFORMING GROWTH FACTOR-β AND SMAD4 REGULATION OF INVASIVE AND METASTATIC BEHAVIOR IN CANCER CELLS

By

Sheng-Ru Shiou

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Cell and Developmental Biology

May, 2006

Nashville, Tennessee

Approved:

Professor R. Daniel Beauchamp

Professor Stephen R. Hann (chair)

Professor Raymond N. DuBois

Professor D. Brent Polk

Professor Ann W. Richmond

Professor Douglas E. Vaughan

To my parents, sisters,

and

Richard Ho, infinitely supportive

ACKNOWLEDGEMENTS

I am grateful to those persons who gave valuable advice and intellectual input on my work; their help undoubtedly contributes to whatever values this dissertation many have. First, I would like to thank my advisor and mentor, Dr. R. Daniel Beauchamp, for his continuous support and guidance during my graduate study and the freedom he gave me to choose research projects. I am also grateful to my committee chair, Dr. Stephen R. Hann and the committee members, Drs. Raymond DuBois, Brent Polk, Ann Richmond, and Douglas Vaughan for their detailed and helpful criticisms of various parts of my work. I am also grateful to Drs. Pran Datta and Punita Dhawan, who generously provided me with reagents and technical supports. I would like to extend my gratitude to Dr. Natasha Deane and Cindy Kanies for their valuable suggestions on dissertation and manuscript preparation and lively discussions that have made my research life joyful. Finally, I thank Jinghuan Janey Zi and Young-Jin Gi, who helped to conduct my experiments when I was busy writing my manuscripts and dissertation. Their and others' friendship has provided great happiness in my life and contributed great enjoyment to my years at Vanderbilt.

I especially want to thank my family, whose tremendous love is with me in whatever I pursue. I also wish to thank Richard Ho, who has been such a positive force during the past years. Their encouragement and support in my life are immeasurable.

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LIST OF ABBREVIATIONS

APC	adenomatous polyposis coli
BMP	bone morphogenetic protein
bp	base pair
CRC	colorectal cancer
DN	dominant negative
DNIIR	dominant negative transforming growth factor-β type II receptor
ECM	extracellular matrix
EMT	epithelial-to-mesenchymal transition
MMP	matrix metalloproteinase
nt	nucleotide
PAI	plasminogen activator inhibitor
PMSF	phenylmethylsulfonic fluoride
SDS	sodium dodecyl sulfate
TCF	T cell factor
TGF-β	transforming growth factor-β
TβRI	transforming growth factor-β type I receptor
TβRII	transforming growth factor-β type II receptor
uPA	urokinase plasminogen activator
uPAR	urokinase plasminogen activator receptor
VEGF	vascular endothelial growth factor
Wnt	wingless

CHAPTER I

INTRODUCTION

TGF-β signaling

TGF-β signaling pathway

TGF-β ligands and ligand binding proteins

The Transforming Growth Factors-beta (TGF-β) superfamily of cytokines are multifunctional molecules that play pivotal roles in the control of cell proliferation, differentiation, cell adhesion, cell motility, apoptosis, extracellular matrix production, skeletal development, haematopoiesis, inflammatory responses, and wound healing (reviewed in [1, 2]). This superfamily is composed of two subfamilies of cytokines; the TGF-β/Activin/Nodal subfamily and the Bone Morphogenic Protein (BMP)/Growth and Differentiation Factor (GDF)/Muellerian Inhibiting Substance (MIS) subfamily [2] (Fig. 1). There are three mammalian TGF-β isoforms, TGF-β1, TGF-β2, and TGF-β3, which are encoded by distinct genes on different chromosomes and are expressed in both a tissue-specific and in a developmentally regulated fashion [3]. Of these isoforms, TGF-β1 (TGF-β) is most frequently up-regulated in human neoplasia [4, 5] and is the focus of studies in our laboratory.

TGF-βs are synthesized as pro-cytokines that are cleaved into corresponding amino-terminal pro-peptides, LAPs (latency-associated proteins) and carboxy-terminal fragments (the mature cytokines) [6]. Unlike most other cytokines, mature TGF-βs remain non-covalently

associated with LAPs in forms that are not recognized by its signaling receptors. The physiological activation process of latent TGF-βs is probably a multi-step process involving proteolysis by protease systems such as the urokinase plasminogen activator (uPA) system [7, 8]. The activation of TGF-β by uPA has been demonstrated in several tissues and cell types especially in pathogenic contexts [7, 8].

Ligand access and activity of the signaling receptors is also regulated by various families of diffusible proteins known as ligand binding traps [9] including Noggin, Chordin, and Follistatin (Fig. 1). Follistatin is a soluble secreted glycoprotein that suppresses Activin activity through inhibition of Activin binding to its receptors [10]. Although originally identified as an Activin-binding protein, Follistatin was shown to inhibit all aspects of bone morphogenetic protein (BMP) activity in early Xenopus embryos [11]. Interestingly, Follistatin was found to noncompetitively inhibit the BMP receptor function by forming a trimeric complex with BMP and its receptor [11]. Unlike Follistatin, Noggin [12] and Chordin [13] function as BMP antagonists by interfering with BMP binding to its cell-surface receptor.

TGF-β signal transduction

TGF-β ligands signal through various type I and II transmembrane serine/threonine kinase receptors (Fig. 1). A type II receptor is necessary for specific ligand binding [2, 14]. Once bound to ligands, type II receptors recruit, bind, and transphosphorylate type I receptors, thereby stimulating type I receptor protein kinase activity. Following ligand binding and receptor activation, information from the tissue microenvironment to the cell nucleus by these growth factors is then transmitted by specific receptor-associated Smad (R-Smad). Eight members of the vertebrate Smad family of protein have been identified and are referred to as

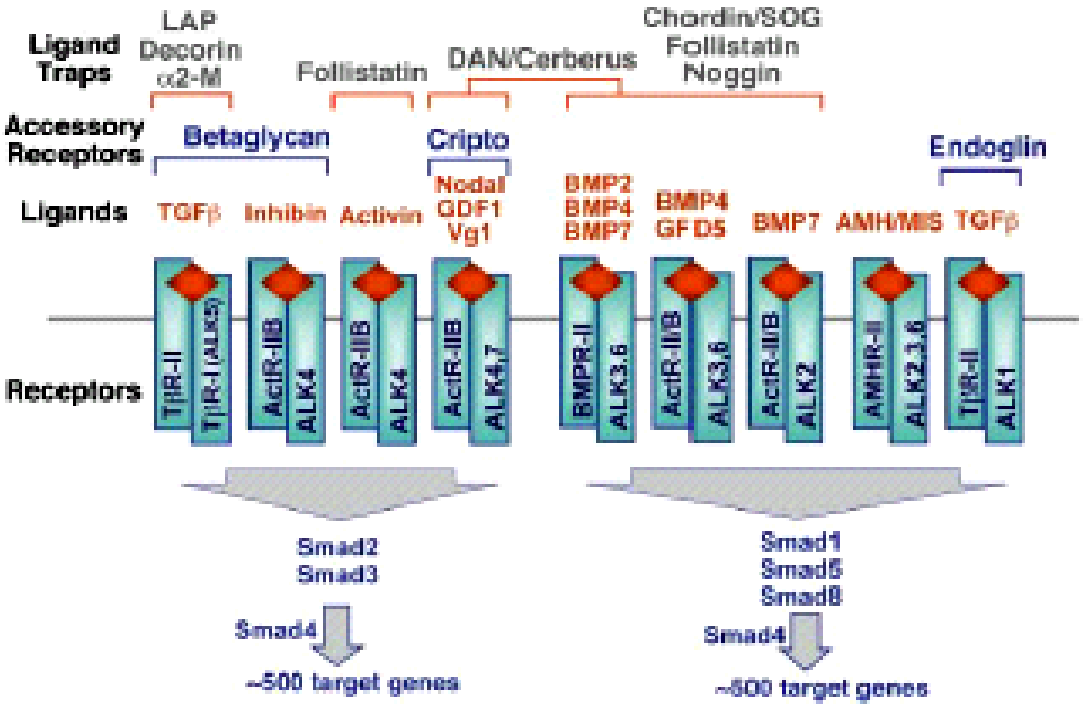

Figure 1. Schematic relationship describing TGF-β ligands, ligand binding traps, accessory receptors, and the type I and type II receptors. Downstream R-Smads 1, 2, 3, 5 and 8 are grouped based on their signaling specificity. Smad2 and 3 mediate TGF-β and activin signaling whereas Smad1, 5, and 8 transduce BMP signaling. Figure from Shi Y. and Massague J., Cell. 2003. 113:685-700.

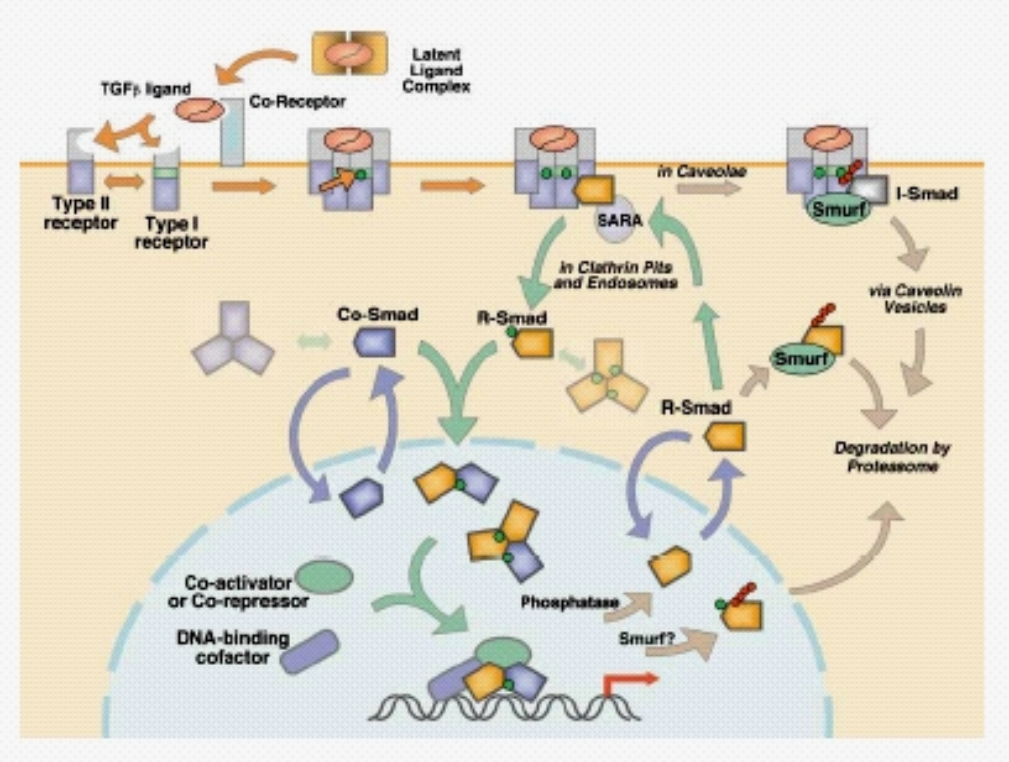

Figure 2. Schematic diagram of TGF-β signaling from cell membrane to the nucleus. TGF-β family ligands bind to type II receptors which then recruit and activate type I receptors by phosphorylation. Subsequently, R-Smads activated and phosphorylated by type I receptors complex with the Co-Smad (Smad4) and then translocate into the nucleus, where they regulate the expression of TGF-β target genes in a cell-type specific manner. The arrows indicate signal flow and are color coded: orange for ligand and receptor activation, gray for Smad and receptor inactivation, green for Smad activation and formation of a transcriptional complex, and blue for Smad nucleo-cytoplasmic shuttling. Phosphate groups and ubiquitin are represented by green and red circles, respectively. Figure from Shi Y. and Massague J., Cell. 2003. 113:685-700.

Smad1 through Smad8 [2] (Fig. 1). As a general rule, Smads1, 5, and 8 transduce signals from the bone morphogenetic proteins (BMPs) through BMP-specific receptors whereas Smads2 and 3 transduce signals downstream of TGF-β and Activin signals [2, 15]. After phosphorylation by a type I receptor, R-Smads associate with the common-Smad (Co-Smad), Smad4, and translocate to the nucleus to regulate transcription of a multitude of TGF-β target genes, independently or in association with DNA-binding partners (Fig. 2). Smad6 and Smad7 are inhibitory Smads (I-Smads) that counteract Smad-mediated signaling by interfering with type I receptor-mediated R-Smad phosphorylation [2]; in general, Smad6 preferentially inhibits BMP signaling whereas Smad7 can inhibit both TGF-β and BMP signaling.

Although Smad2, Smad3, and Smad4 comprise the core Smad-dependent TGF-β signaling pathway, accumulating evidence suggests that Smad-independent TGF-β signaling may also occur in cells. Smad-independent TGF-β signaling may be mediated through the mitogen-activated protein kinase (MAPK), Rho guanosine triphosphatases, PI-3 kinase/Akt, and protein phosphatase 2A signaling pathways (reviewed in [16]). However, activation of these pathways appears to be cell context-dependent. For example, TGF-β activation of the Ras/Raf/MAPK pathway required expression of oncogenic Ras in selected cells [17].

TGF-β in cancer

TGF-β has been considered both a tumor suppressor and tumor promoter. Generally, TGF-β may elicit anti-tumorigenic effects at early stages during carcinoma development, while exhibiting tumor promoting effects in later stage cancer (Fig. 3).

Tumor suppression by TGF-β

Most pertinent to our understanding of the role of TGF-β in carcinoma development is the fact that TGF-β is a potent inducer of growth inhibition [18-20] and apoptosis [21] in several cell types, primarily epithelial lineages. Virtually almost all epithelial-derived tumors (>85% of all human cancers) lose the growth-inhibitory response to TGF-β [22]. In some cancers, including colon and pancreatic cancers, mechanisms for resistance such as functional inactivation of the Smad proteins (predominately Smad4) or the TGF-β receptors (predominately TβRII) [22] are well defined. However, in other cancers such as breast, lung, and prostate, the mechanisms for resistance to TGF-β's growth inhibitory effect remain poorly understood. Therefore, abrogation of TGF-β signaling leading to a loss of growth inhibition is considered one of the mechanisms for the loss of protection against tumor progression. The role of TGF-β as a tumor suppressor is further highlighted by the *in vivo* studies involving genetic manipulations of expression of components of TGF-β signaling pathway in tumor cells [23] [24]. For example, overexpression of a TGF-β type II receptor reduced the tumorigenicity and metastatic potential of K-ras-transformed thyroid cells [23], whereas expression of dominant-negative forms of TβRII in mammary gland increased tumor formation induced by a chemical carcinogen in mice [24].

One key event that leads to TGF-β-induced growth arrest is the induction of the CDK inhibitors, p15INK4B [25] and p21CIP1 [26]. p21CIP1 interacts with complexes of CDK2 and cyclin A or cyclin E thereby inhibiting CDK2 activity and preventing progression of the cell cycle [27]. By contrast, p15INK4B interacts with and inactivates CDK4 and CDK6 or associates with cyclin D complexes of CDK4 or CDK6. The latter interaction not only inactivates the catalytic activity of these CDKs but also displaces p21CIP1 or the related p27KIP1 from these

complexes, allowing them to bind to and inactivate the CDK2 complexes with cyclin A and E [27, 28]. Additional mechanisms underlying TGF-β-mediated growth arrest include indirect suppression of c-Myc [29] and CDK4 [30] protein expression.

Programmed cell death or apoptosis is a key cellular response that controls cell numbers. TGF-β has been shown to induce apoptosis in several cell types [21, 31] and in a number of cancers such as breast, gastric, hepatic and prostate cancers [31]. TGF-β-induced apoptosis has been shown to be mediated by the Smad-dependent pathway [32, 33]. However, the inhibitory Smad, Smad7, promotes TGF-β–induced apoptosis in prostate carcinoma cells [34]. There are also studies showing involvement of Smad-independent pathways in this TGF-β action [35]. For example, Daxx adaptor protein, a protein associated with the Fas receptor that mediates activation of Jun amino-terminal kinase (JNK) and programmed cell death induced by Fas, has been proposed to mediate TGF-β–induced apoptosis through interaction with a TGF-β type II receptor [35]. The downstream events that lead to apoptosis induced by TGF-β include down-regulation of anti-apoptotic factors such as Bcl-XL [36] and up-regulation of pro-apoptotic factors such as caspases 3 and 8 [37].

TGF-β enhances tumor progression

Solid tumors exhibit lethal effects by invading into surrounding tissues and metastasizing to distant sites in the body. Clinically, elevated TGF-β has been detected in late stage cancer and is frequently associated with advanced disease [31]. TGF-β displays the capacity to promote tumor progression in cancers through various mechanisms [38-42]. Among these functions, TGF-β has an important role in the regulation of metastasis and epithelial-to-mesenchymal transition (EMT) in cancers [43-45]. Epithelial-to-mesenchymal transition (EMT) has been

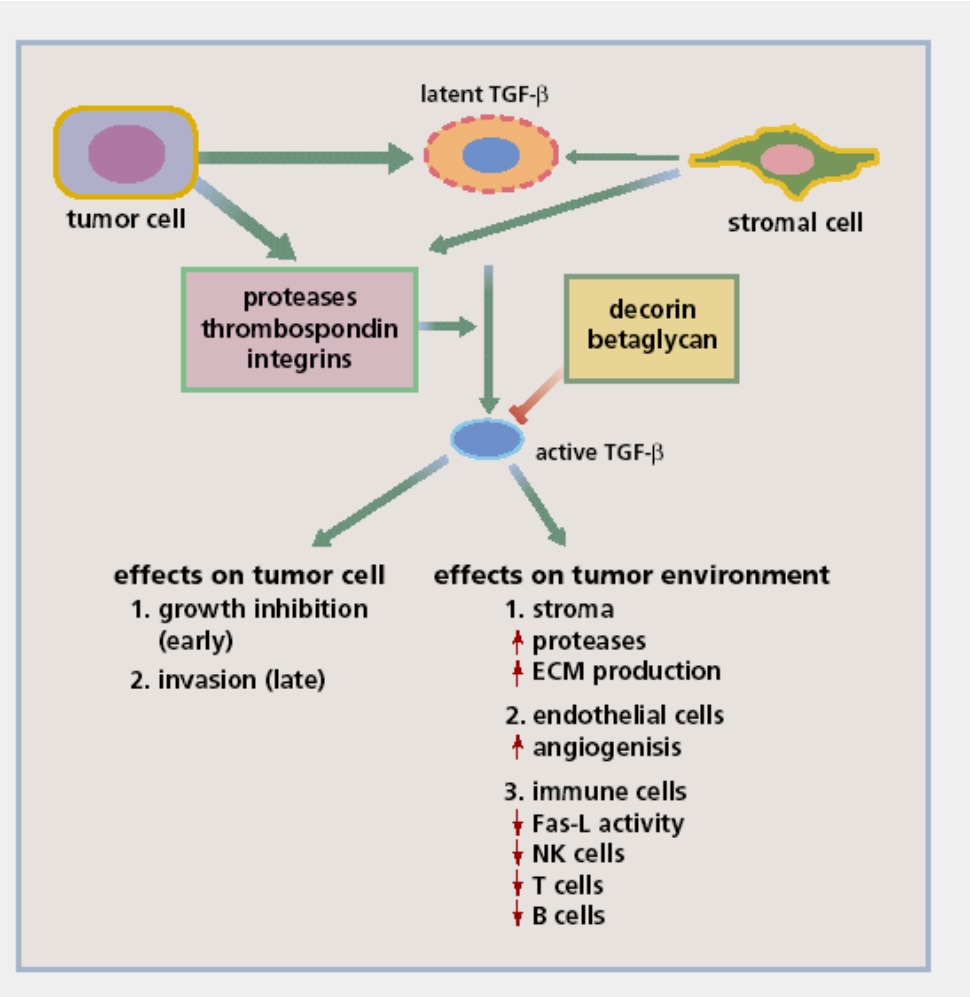

Figure 3. Activation of and dual roles for tumor and stromal TGF-β in tumorigenesis through interaction with the tumor cell and its environment. Both tumor and stromal cells secrete elevated levels of TGF-β that are self-perpetuating via a cycle of enhanced protease activation of latent TGF-β, auto-induction of TGF-β gene activity. Most TGF-β activities, apart from the growth inhibition of early tumor cells, promote tumor progression. ECM, extracellular matrix; Fas-L, Fas ligand. Figure from Derynck, Rik, et al., Nat. Genet. 2001. 29:117-129.

shown to correlate with increased invasive and metastatic capacity of tumor cells (reviewed in [46]) during cancer progression. TGF-β was described as an inducer of EMT first in normal mammary epithelial cells [41] and later in tumors. This TGF-β's function was demonstrated in a study showing that ectopic TGF-β expression keratinocytes in transgenic mouse appeared to enhance progression of the cells toward a more malignant spindle-cell phenotype in advanced lesions after long-term treatments of a chemical carcinogen [47]. Moreover, TGF-β also promotes the EMT that accompanies invasive and metastatic growth of transformed cells [48, 49]. In a highly metastatic mesenchymal mouse colon carcinoma line (CT26), overexpression of a dominant-negative TGF-β type II receptor (DN-TβRII) blocked TGF-β signaling and induced reversal of EMT in these cells. Expression of the DN-TβRII also inhibited cell invasion and abolished metastasis formation by CT26 cells. In addition, after undergoing EMT, tumor cells may acquire altered TGF-β responsiveness such as resistance to the tumor-suppressor effects of TGF-β [50]. Thus although TGF-β can inhibit tumor growth, there is increasing evidence that TGF-β secretion by tumor cells and/or stromal cells within the peri-tumoral microenvironment contributes to tumor maintenance and progression [43, 51, 52].

TGF-β exhibits activity that directly contributes to metastatic growth [53] [54]. In the metastatic 4T1 breast cancer cells expression of a dominant-negative truncated type II receptor diminished TGF-β signaling and significantly restricted the ability of 4T1 cells to establish distant metastases [53]. The same group also showed that a soluble Fc:TGF-β type II receptor fusion protein (Fc:TβRII) inhibited breast cancer metastases in transgenic and xenograft mouse models. Similarly, Yang et al [54], developed transgenic mice expressing a TGF-β antagonist of the soluble Fc:TβRII fusion protein class, under the regulation of the mammary-selective MMTV-LTR promoter/enhancer. These mice were resistant to the development of metastases at

multiple organ sites when compared with wild-type controls, both in a tail vein metastasis assay using isogenic melanoma cells and in crosses with the MMTV-neu transgenic mouse model of metastatic breast cancer.

Cancer cells can proliferate independently of exogenous mitogenic growth signals through either overexpression of the mitogenic growth factors or constitutive activation of mitogenic signaling pathways. TGF-β can stimulate the proliferation of a number of epithelial-derived cancer cell lines, including colon, pancreatic, and prostate cancer cells by, in part, increasing the expression of mitogenic growth factors or their receptors (reviewed in [22]). In addition, TGF-β can activate the proliferative Ras/Raf/MAPK pathway in a Smad-independent manner in cells that express oncogenic Ras protein [55].

TGF-β is one of the several cytokines that coordinate to regulate angiogenesis, which is frequently associated with deregulated expression of vascular endothelial growth factor (VEGF). The presence of TGF-β in the microenvironment of tumor sites thus might indirectly provide solid tumors with nutrients and oxygen supplies though modulating angiogenesis. TGF-β has been shown to potentiate the hypoxic response in human HT-1080 fibrosarcomas [56] and increases *VEGF* transcription by enhancing the DNA-binding activity of the transcriptional factors, AP-l and HIF-1, and may. Moreover, TGF-β-enhanced liver metastatic potential of human pancreatic cancer cell lines, Capan-2 and SW1990 was associated with VEGF production [57].

Although there are multiple mechanisms by which cancer cells evade immune responses, a major mechanism is escaping from cancer cell-mediated immune response via secretion of TGF-β [58]. This immuno-suppressive activity of TGF-β is suggested by the following observations showing that a highly immunogenic tumor transfected with a murine TGF-β cDNA

escaped immune surveillance and formed tumors in animals [59] and anti-cancer cell immune responses can be augmented by blocking the TGF-β signaling pathway in T cells [60].

Autocrine and paracrine TGF-β

Cancer cells express receptors enabling them to respond to a variety factors derived from the non-tumor cells in tumor and stromal microenvironments [61]. Additionally, cancer cells can synthesize and secrete various growth factors that contribute to malignant tumor growth through autocrine mechanisms [40] (Fig. 2). The impact of tumor-derived TGF-β on cell invasion and metastasis through autonomous mechanisms has been documented [40, 49]. For example, disruption of autocrine TGF-β signaling by a dominant-negative type II receptor (DNIIR) inhibited the invasive and metastatic potential of mammary and colon carcinoma cells [49]. This effect was attributed to the prevention of autocrine TGF-β-induced epithelial-to-mesenchymal transition (EMT), a process believed to promote tumor cell migration and invasion [62]. Furthermore, inhibition of autocrine TGF-β signaling by over-expression of a soluble TGF-β type III receptor, which antagonizes TGF-β activity caused inhibition of proliferation and induction of apoptosis [40]. These data demonstrate a role for autocrine TGF-β in regulation of cancer cell behavior. The mechanism by which autocrine TGF-β may selectively contribute to tumor cell behavior was explored in the first part of my study using the breast cancer cell line MDA-MB-231 [63]. MDA-MB-231 cells express MMP-9 and uPA [51]. Elevated MMP-9 and uPA activity has been linked to metastatic growth of cancer cells and poor patient outcome [64-66]. We hypothesized that autocrine TGF-β could function as a tumor promoter through regulation of MMP-9 or uPA activity in these cells.

Development of TGF-β signaling inhibitors for cancer therapy

Deregulation of TGF-β has been implicated in the pathogenesis of a variety of diseases, including cancer and fibrosis disorders [22, 67]. The role of TGF-β in cancer biology is complex and involves aspects of tumor suppression as well as tumor promotion. The ability of TGF-β to potently inhibit the proliferation of epithelial, endothelial and haematopoietic cell lineages is central to its tumor-suppressive function [68]. However, as tumors progress, they often become refractory to TGF-β-mediated growth inhibition and even overexpress TGF-β, which in turn has a marked impact on the biology of the tumor cells themselves and creates a tumor micro-environment that is conducive to tumor growth and metastasis. Therefore, antagonism of TGF-β signaling will potentially have an impact on physiological and pathological activity of TGF-β in normal and tumor cells.

Large-molecule TGF-β signaling inhibitors

The more advanced TGF-β signaling antagonists in clinical development are large molecules, including monoclonal antibodies [69, 70] and antisense oligonucleotides [71] that target the TGF-β protein molecule and the TGF-β mRNA, respectively. Initial efforts to develop the large-molecule TGF-β signaling antagonist approaches because selective inhibition of the receptor kinase in the heteromeric receptor and Smad signaling complex was not thought to be achievable. Therefore, the large-molecule antagonists of TGF-β signaling are more advanced than the small-molecule antagonists. Nevertheless, delivery issues for the large-molecule TGF-β antagonists [72], might limit their application to particular disease states. The ability of TGF-β to signal in an autocrine mode could also limit the effectiveness of antibody therapies in solid tumours.

Small-molecule TGF-β receptor kinase inhibitors

The extensive knowledge surrounding TGF-β receptor kinase-dependent Smad2/Smad3 phosphorylation as an early event of TGF-β signal transduction has focused the development of Small-molecule TGF-β receptor kinase inhibitors as a therapeutic target [73, 74]. These inhibitors have key functional centers that potently bind to the kinase-domain active sites of the TGF-β receptors and thus act as ATP-competitive compounds [74]. Although the development of small-molecule kinase inhibitors may overcome the delivery and tissue-penetration issues, these inhibitors are inherently less selective than antisense or antibody therapies. In addition, it is anticipated that the pharmacokinetic properties of antibody and small molecule kinase inhibitors will result in considerable differences in the duration of TGF-β signaling modulation. In general, antibody therapy will have a prolonged duration of activity relative to the small-molecule kinase inhibitors. Thus, the differences in mechanisms of action and pharmacokinetic properties make future combination therapies of small-molecule TGF-β receptor kinase inhibitors and neutralizing antibody therapies very attractive.

Although previous studies and our work have shown the therapeutic potential of inhibiting TGF-β signaling and the efficacy of the TGF-β receptor kinase inhibitor in suppressing tumor cell invasion, respectively, there are many reports that have highlighted the importance of the tumor-suppressive role of TGF-β, and how the absence of TGF-β signaling can exacerbate tumorigenesis [24, 75]. This underscores the need to carefully define patient populations in which the tumor promoting role of TGF-β signaling predominates. Moreover, it will be valuable for investigating the molecular nature and stage of tumors that are susceptible to TGF-β antagonism, and, conversely, for identifying the stages at which the tumor-suppressive role of TGF-β signaling remains operative.

Smad transcriptional regulation of gene expression

Smad proteins consist of three domains, namely the highly conserved amino-terminal (MH1) and carboxy-terminal (MH2) domains, and the intervening linker region with variable length and sequence [2]. The MH2 domain displays transcriptional activity in a heterologous fusion with a GAL4 DNA binding domain in transcriptional studies. Smads (most R-Smads) and the Co-Smad (Smad4) bind to DNA in a sequence-specific manner. The minimal Smad binding element (SBE), was initially identified as the optimal DNA binding sequence 5'-AGAC-3' [76]; however most naturally occurring DNA sequences contains an extra base "C" at the 5' end. In addition to the SBE, certain G/C-rich sequences have been reported to be Smad1, Smad3, and Smad4 binding elements [77, 78].

TGF-β stimulation leads to positive and negative regulation of several hundred target genes. Both activation and repression of gene expression use the same set of activated Smad proteins, suggesting that Smads must cooperate with each other and/or with other DNA binding partners to elicit specific transcriptional responses. A general hypothesis for how cells respond to TGF-β signals posits that Smad access to target genes and the recruitment of transcriptional coactivators or corepressors to such genes depends on the availability of specific partner proteins in different cell context and conditions that affect the cell at the time of TGF-β stimulation. Indeed, the MH2 domain of both R-Smads and Smad4 has been shown to interact with the transcriptional coactivators CBP/p300 [79] and Smif [80] as well as the co-repressors Ski and SnoN. Ski and SnoN are two highly conserved members of the Ski family of proto-oncoproteins [81]. Ski and SnoN antagonize TGF-β signaling through direct interactions with Smad4 or the

R-Smads. The mechanisms of Ski-mediated repression of TGF-β signaling are primarily attributed to transcriptional modulation through recruitment of the nuclear transcriptional corepressor (N-CoR) and histone deacetylase (HDAC) and interference of Smad-mediated binding to the transcriptional coactivator, p300/CBP [81]. Thus, by functioning in conjunction with various positive and negative (co)activators or (co)suppressors, Smad proteins can mediate transcriptional activation or repression depending on their associated partners.

The roles of Smad in cancer

Smads are important intracellular effectors in mediating TGF-β-induced growth inhibition and apoptosis [32, 33]. Loss of the normal growth inhibitory response to TGF-β has been linked to functional inactivation of type II TGF-β receptors (TβRII) or Smad proteins. The most commonly lost Smad mediator in cancers is Smad4. Functional inactivation of Smad4 was detected in pancreatic (~50%) [82] and colorectal (20-30%) [83, 84] cancer and in 80% of adenocarcinomas of the small bowel [84]. Furthermore, loss of Smad 4 expression is correlated with liver metastasis and poor prognoses in colon cancer patients [83, 84]. Smad4 displays tumor suppressive functions primarily through mediating TGF-β-induced growth inhibition and apoptosis. Recent evidence suggests that Smad4 has additional tumor suppressive functions [85-87]. For example, expression of Smad4 in Smad4- and E-cadherin-deficient colon carcinoma cells resulted in reversal of epithelial-to-mesenchymal transition (EMT), increased E-cadherin expression, and the loss of tumorigenesis of the cells in nude mice [85-87]. In addition, these phenotypic changes were associated with reduced protein expression of prognostic markers such as urokinase plasminogen activator (uPA) and plasminogen-activator-inhibitor-1 (PAI-1) [38, 66, 87]. In a separate study, restoration of Smad4 in pancreatic carcinoma cells suppressed tumor

formation *in vivo* [88]. Further, the Smad4-expressing cells produced less angiogenic factor, vascular endothelial growth factor (VEGF), and developed to smaller non-progressive tumors with reduced vascular density when compared with Smad4-deficient parental cells. Collectively, these data define novel mechanisms of tumor suppression by Smad4.

Paradoxically, the prevailing evidence suggests that induction of EMT by TGF-β is mediated through the canonical Smad2/3 and Smad4 transduction pathway. In a study led by Valcourt et. al [89], either dominant-negative Smad3 or dominant-negative Smad2 could inhibit the EMT response of NMuMG mouse mammary epithelial cells to TGF-β. Expression of a dominant-negative (C-terminally deleted) Smad4 also inhibited EMT [89]. Thus, Smads-elicited tumor suppressive and promoting effects may be dependent on cell context.

Potential for TGF-β signaling-independent roles for Smad4

As described above, expression of Smad4 in Smad4-deficient colon carcinoma SW480 cells reduced the tumorigenicity of the cells [85]. Interestingly, Smad4 expression did not restore *in vitro* growth inhibitory response to TGF-β in the cells, which was probably attributed to low levels of type II TGF-β receptor resulting in deficient TGF-β signaling [85]. These data suggest that Smad4 may elicit tumor suppressive responses independently of TGF-β signaling.

Urokinase plasminogen activator (uPA) – an important microenvironmental component of Cancer

The biological functions of the uPA system

The urokinase plasminogen activator (uPA) system is central to a spectrum of biologic processes including fibrinolysis, inflammation, atherosclerotic plaque formation, matrix

remodeling, and growth factor activation [7, 90, 91]. The primary function of uPA is converting the inactive pro-enzyme plasminogen into plasmin (Fig. 4). Plasmin is a serine protease capable of degrading components of the basement membrane and extracellular matrix (ECM). Plasmin also triggers activation of ECM-degrading proteases such as metalloproteinases [64] (Fig. 4) that have been shown to be linked to the metastatic growth of cancer [38]. Urokinase plasminogen activator and plasmin are also involved in the activation or liberation of latent growth factors such as vascular endothelial growth factor (VEGF) [92] and TGF-β [93, 94] that play an important role during tumorigenesis.

Through binding to it receptor, uPA can activate intracellular signaling pathways such as the extracellular-signal-regulated kinase-1 and -2 (ERK1/2), p38, and focal adhesion kinase (FAK) pathways that are important for proliferation and migration [95, 96] and increase the activity or expression of some transcription factors such as AP-1, c-Jun, and c-Myc in certain cellular contexts [95]. UPAR-mediated uPA effects can be independent of uPA proteolytic activity. Both pro-uPA and uPA bind with high affinity to uPAR. Moreover uPA, pro-uPA, and protease activity-defective uPA have been shown to be capable of inducing cell migration comparatively in selected cells [97, 98].

Besides transducing signals from uPA, uPAR can regulate migration, cell attachment and signaling transduction through interaction with vitronectin and integrin [91, 96, 99]. Since uPAR lacks transmembrane and cytoplasmic domains and is anchored in the plasma membrane by a glycosyl phosphatidyiinosital (GPI) moiety, other membrane partners are probably required to transduce uPAR-mediated signal transduction across the plasma membrane.

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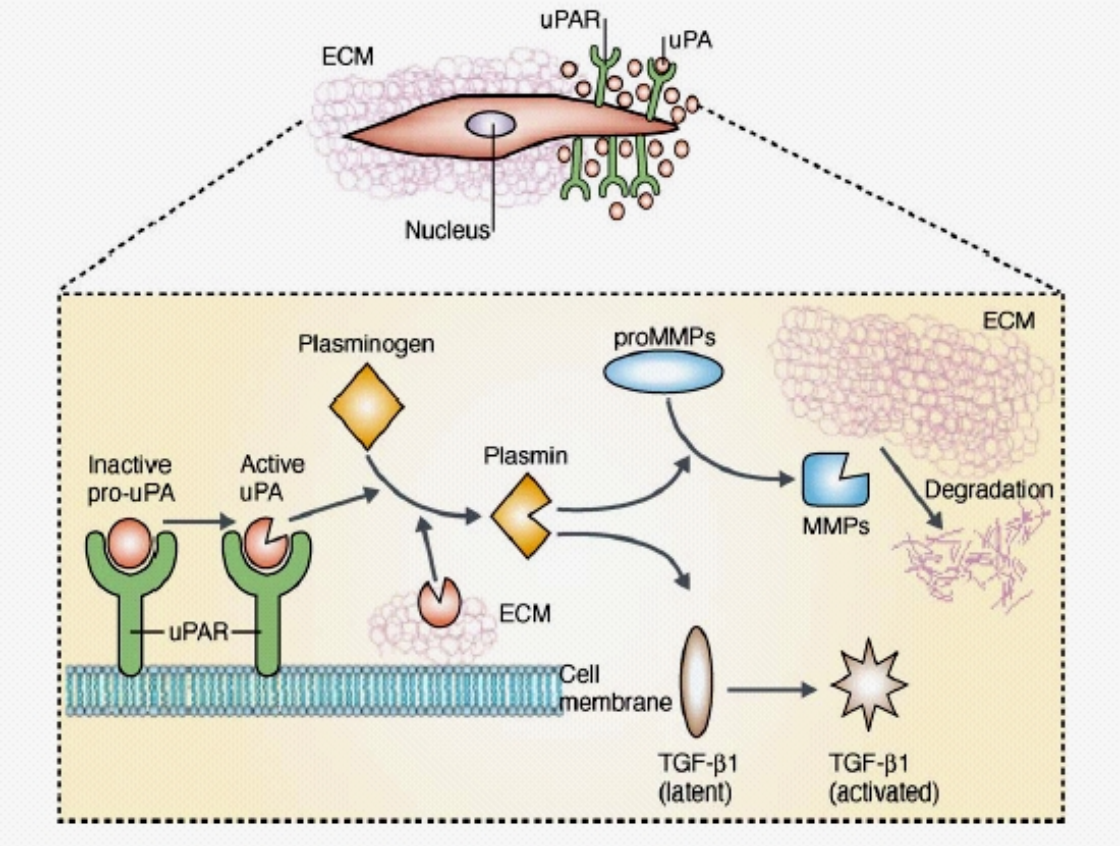

Figure 4. Diagram of uPA actions. At the leading edge of migrating cells, the urokinase receptor (uPAR) binds inactive urokinase (pro-uPA), which is then converted to active uPA. Active uPA proteolytically converts the inactive zymogen plasminogen to active plasmin, which then breaks down ECM components or activates latent growth factors such as transforming growth factor-β1 (TGF-β1). Plasmin can also degrade the ECM indirectly through activation of pro-matrix metalloproteinases (MMPs). Figure from Duffy M. J., et al., 2004. Clin. Biochem. 37:541-548.

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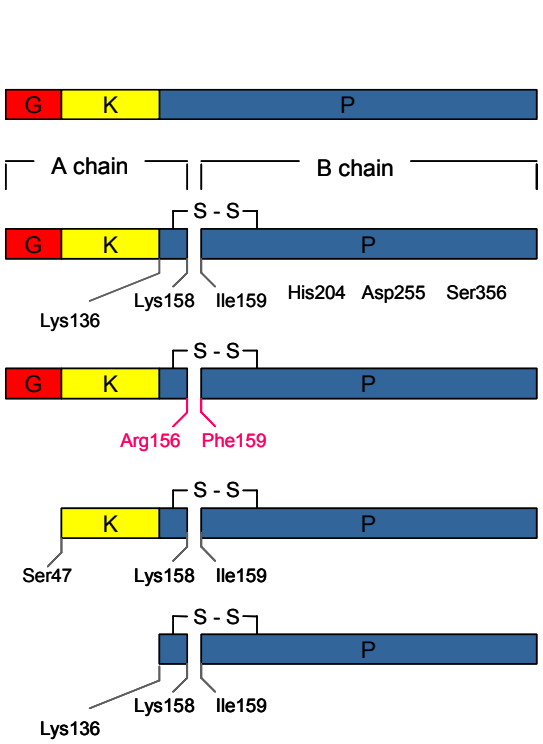
The key components of the uPA system

Urokinase Plasminogen activator (uPA)

Urokinase plasminogen activator was first identified in urine and later found to be expressed in vascular endothelial, smooth muscle, fibroblast, monocyte/macrophage, and epithelial cells as well as in tumors of different origins [91]. This protein is secreted by cells as a single-chain zymogen (pro-uPA) with a molecular weight of 54 kD and has three functional domains: the N-terminal "growth factor-like" domain responsible for receptor binding, the central kringle domain that interacts with the uPA inhibitors, plasminogen activator inhibitor-1 and -2 (PAI-1 and PAI-2), and the C-terminal catalytic domain with serine protease activity with a specific amino acid triad, His^{204}, Asp^{255}, and Ser^{356} in the active center [91] (Fig. 5). Activation of pro-uPA involves cleavage of peptide bond between Lys158 and Ile159 by proteases including plasmin, elastase, and cathepsin B [91, 99], resulting in the formation of a two-chain uPA molecule linked by a disulfide bond. A low-molecular-weight uPA has been detected in human urine [100] and plasma from patients with acute pancreatitis [101]. The low-molecular-weight form can be generated after further cleavage of the Lys135-Lys136 bond by plasmin in the region between the kringle and protease domains (Fig. 3), and its proteolytic



	MW	Relative activity
Single-chain pro-uPA	54 kDa	1
Two-chain active uPA	54 kDa	>200
Two-chain inactive uPA	54 kDa	0
Two-chain active uPA	50 kDa	>200
Two-chain active uPA (Most abundant LMW uPA)	32 kDa	>200

Figure 5. Fragments of urokinase plasminogen activator generated after proteolytic processing. Activation of pro-uPA involves cleavage of the peptide bond between Lys158-Ile159 by proteases such as plasmin, elastase, and cathepsin B, resulting in the formation of a two-chain uPA molecule linked by a disulfide bond. The low-molecular-weight form can be generated after further cleavage of the Lys135-Lys136 bond by plasmin in the region between the kringle and protease domains. G: growth factor-like domain; K: kringle domain; P: protease domain; MW: molecular weight, LMW: low molecular weight.

activity is comparable to the activity of the full-length two-chain uPA [100, 102]. Although the single-chain pro-uPA is a zymogen (pro-uPA), it has some intrinsic activity [103] and thus is able to convert its substrate, plasminogen, to plasmin. Therefore, pro-uPA-mediated generation of plasmin will facilitate activation of itself, resulting in cyclic activation of this system.

Urokinase plasminogen activator receptor (uPAR)

The 55-60 kD uPAR protein was first identified as a uPA-binding receptor and was later found to be an adhesion receptor for vitronectin, a component of ECM [104] as well. Coincident binding of uPA to uPAR and plasminogen to plasminogen receptors on the cell surface strongly increases local concentrations of uPA and plasminogen, thereby accelerating conversion of plasminogen to plasmin by uPA. Both pro-uPA and uPA bind to uPAR with high affinity [97, 98]. While pro-uPA has low intrinsic activity for conversion of plasminogen to plasmin, uPAR plays an important role in facilitating the activation of pro-uPA by plasmin and activation of plasminogen by uPA.

Plasminogen activator inhibitors (PAI-1 and PAI-2)

Plasminogen activator inhibitor-1 and -2 (PAI-1 and PAI-2) are the principle inhibitors of uPA activity. Both PAI-1 and PAI-2 inhibit uPA by forming a complex with active uPA at a 1:1 ratio [105]. PAI-1 predominates as an inhibitor of uPA, as it reacts with uPA faster than PAI-2 [105]. After binding, uPA/PAI complexes are internalized by cells in a process requiring binding of uPA/PAI to uPAR [106]. The process of internalization leads to the degradation of both uPA and PAI-1 while uPAR is recycled to the cell surface [107]. As acceleration of uPA-mediated

cell surface activation of plasminogen depends upon uPA binding to uPAR, the clearance of inactive uPA/PAI-1 complexes and recycling of uPAR, maintains the cells' capacity to bind uPA.

Evidence for a role of the uPA system in cancer progression

High expression levels of components of the uPA system, including uPA and its cell surface receptor (uPAR), have been correlated with poor prognosis in cancers [66, 108]. Overexpression of uPA is observed in carcinomas and metastases of breast, colon, lung, brain, kidney, bladder, liver, and brain cancers [66, 109, 110]. In breast cancer, high levels of uPA in tumor tissues predict poor prognosis and shorter survival [66, 109]. Its role in promoting tumor invasion and metastasis has also been well documented in a number of *in vitro* and *in vivo* studies. In cell cultures, uPA overexpression induced by cytokines promoted cell invasion [38, 111]. Ectopic overexpression of uPA in murine melanoma B16-F1 cells significantly increased metastasize potential of the cells [112]. In the same study, the uPA-overexpressing melanoma cells also exhibited a greater capacity to extravasate from the blood vessels and developed into pulmonary tumors when compared with control cells. Conversely, introduction of antisense uPA reversed the enhanced metastatic and extravasation potential of uPA-overexpressing melanoma cells. Several other studies also showed that inhibition of expression or activity of the components of the uPA system (e.g. using anti-uPA antibodies [113] or synthetic inhibitors of uPA [110, 114, 115]) or disruption the interaction between uPA and uPAR [116, 117] leads to a reduction in the invasive and metastatic capacity of many tumors. These data demonstrate a critical role for uPA in the development of invasive and metastatic growth of tumors. Paradoxically, high levels of the uPA inhibitor, PAI-1, predict shorter patient survival [66, 108]. These observations indicate a more complex role for PAI-1 in tumor progression and

angiogenesis than simple inhibition of uPA. The precise roles for PAI-1 in cancer remain to be explored.

Claudin-1 – a tight junction protein and a potential metastatic transformation modulator in colorectal cancer cells

Claudins in cancer

Claudin family proteins are integral membrane components of tight junctions with molecular weights of ~17–27 kD. At least 20 claudin family members have been cloned and characterized from mouse, rat and human (reviewed in [118]). Tissue-specific expression of claudin proteins allows them to perform specialized cellular functions including maintenance of cell polarity and para-cellular barriers in conjunction with other integral membrane partners, such as occludin and the junctional adhesion molecule-1 (JAM1) (Fig. 6). The preservation of cell polarity by claudins suggests tumor suppressive functions of claudins in epithelial-derived neoplasias. Actually, loss of claudin expression is associated with tumor progression in cancers [119-121]. Paradoxically, overexpression of claudins was also observed in carcinomas and metastases in particular cancer types [122-125]. For example, claudin-4 appears to be over-expressed in ovarian [122] and pancreatic [123] cancer. In some cases, altered subcellular location of claudins was also observed in cancer, including mislocalization of claudin-3 and claudin-4 from the membrane to the cytoplasm in ovarian cancer [122] and of claudin-1 in colon cancer [125]. Thus, deregulated expression and mislocalization of claudin appears to be associated with tumor progression in some cancers; however, its exact roles in cancer development remain unclear.

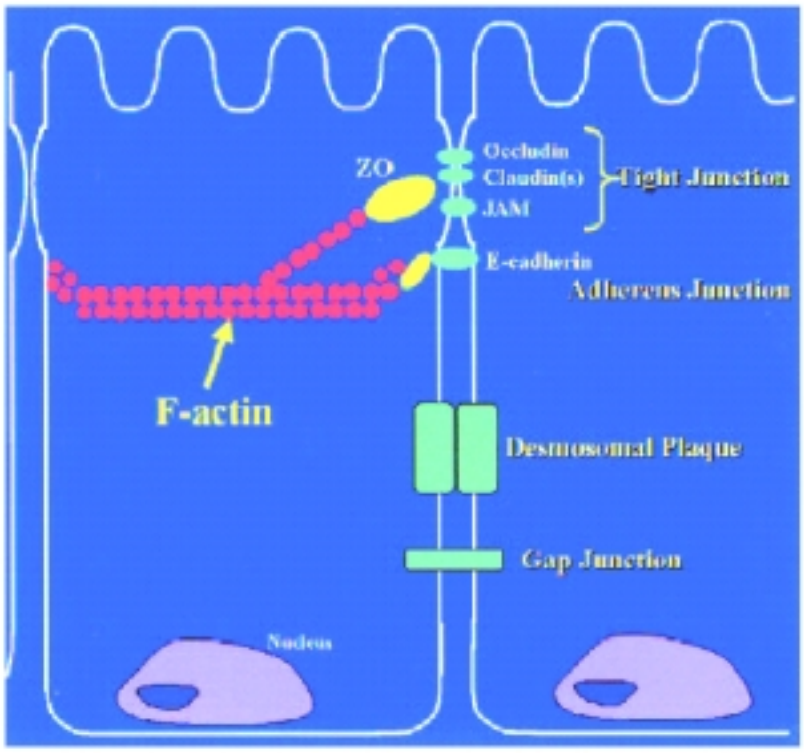


Figure 6. Schematic illustration of intercellular junctions between epithelial cells. The tight junction is located apically on the lateral membrane. The claudins comprise one of the integral membrane components of the tight junction, where they interact with proteins of the ZO family. This interaction in turn links the tight junction to the actin cytoskeleton. Figure from Folpe A. L., et al., Am J Surg Patho,. 2002. 26(12): 1620–1626.

In human colon cancer, claudin-1 expression is increased in carcinomas and metastases [124, 125]. Moreover, genetic manipulation of claudin-1 expression in isogenic variants of primary and metastatic colon cancer cell lines, SW480 and SW620, respectively, implicates a role for claudin-1 in regulating the metastatic potential of these cells [125]. Over-expression of claudin-1 in SW480 cells (non-metastatic) results in hepatic metastases when cells were injected into the spleen of athymic mice, while siRNA mediated knockdown of abundant claudin-1 levels in SW620 cells (metastatic) results in decreased hepatic metastases [125]. The metastatic potential and claudin-1 expression of these cells also correlated with *in vitro* cell invasiveness and anchorage-independent growth. These data suggest a role for claudin-1 in the regulation of invasive and metastatic transformation in colon cancer cells.

Regulation of claudin-1 expression

Snail and Slug regulation of claudin-1 transcription

The Snail family of transcriptional repressors (including Snail and Slug) have been shown to induce epithelial-to-mesenchymal transition (EMT) through downregulation of the adherent junction protein E-cadherin [126]. In promoter studies, Snail and Slug-mediated suppression of E-cadherin transcription was shown to require the E-box *cis*-element in the proximal area of the E-cadherin promoter [126, 127]. Recently, Snail and Slug have been reported to suppress claudin-1 transcription in Madin-Darby canine kidney (MDCK) cells [128] through similar E-box elements in the claudin-1 promoter. In this study, overexpression of Slug and Snail decreased both claudin-1 protein and mRNA levels. In addition, Snail and Slug suppressed a claudin-1 promoter-reporter that contains wild-type E-boxes but not mutated E-

boxes. Direct *in vitro* binding of Snail and Slug to the E-box motifs in the human *claudin-1* promoter were further demonstrated by gel shift assays. These data raise the possibility that deregulation of claudin-1 in cancer may be regulated by Snail and Slug.

Beta-catenin/Tcf regulation of claudin-1 transcription

Beta-catenin is a transcriptional coactivator downstream of Wnt signaling that regulates gene expression through interaction with the transcription factor Tcf (T cell factor). Constitutive activation of β-catenin/Tcf has been implicated in colorectal cancer [129, 130] due to activating mutations in the β-catenin gene or functional loss of adenomatous polyposis coli (APC). APC is one of the components that facilitate β-catenin degradation. Therefore functional loss of APC may lead to activated β-catenin/Tcf signaling [129]. It has been shown that ectopic expression of wild-type APC in APC-deficient colon cancer cells reduced both β-catenin and claudin-1 protein levels [124]. Claudin-1 was recently identified as a probable target of β-catenin/Tcf [124]. Promoter study showed that two putative Tcf4 binding elements in the 5' flanking region of the human *claudin-1* promoter are required for claudin-1 promoter activation induced by β-catenin/Tcf [124], further supporting a role for β-catenin/Tcf in the transcriptional regulation of claudin-1. As β-catenin/Tcf is frequently activated in colon cancer [130] and claudin-1 contributes to metastatic transformation of colorectal cancer cells [125], active Wnt pathway may be involved in stimulation of claudin-1, thereby facilitating metastatic growth of CRC.

Hypothesis

Cancer cells respond to a variety of steroid and polypeptide hormones and growth factors derived from non-tumor cells in tumor microenvironment [61]. Additionally, cancer cells can

synthesize and secrete growth factors that stimulate tumor progression through autocrine mechanisms [40]. Although the mechanisms underlying paracrine TGF-β regulation of tumor behavior is well documented, autocrine TGF-β regulation of tumor cell behavior is less well understood. TGF-β has been shown to regulate the invasiveness and metastasis of carcinomas by stimulating the production of ECM-degrading enzymes [38]. The human metastatic breast cancer cells, MDA-MB-231, have active autocrine TGF-β signaling [63] and express both MMP-9 and urokinase plasminogen activator (uPA) [51]. These findings raised the hypothesis that tumor cell-produced TGF-β regulates tumor behavior through regulation of MMP-9 or uPA activity in an autocrine manner. Results from my studies show that the invasiveness of MDA-MB-231 cells is correlated with autocrine TGF-β signaling. Inhibition of autocrine TGF-β signaling attenuated basal uPA secretion but had no effect on basal MMP-9 production or activity. Furthermore, inhibition of autocrine TGF-β signaling decreased uPA production And autocrine TGF-β regulation of uPA required Smad4 protein expression. With these findings, we conclude that tumor cells can be self-sufficient in promoting their own invasive growth by producing TGF-β, which in turn maintains basal uPA production that is critical for the invasive growth of these cells.

Smad4 was initially regarded as a tumor suppressor due to its role in mediating TGF-β-induced antiproliferative responses [87, 88]. Recent evidence suggests additional tumor suppressing functions of Smad4 [85, 87, 88]. Loss of Smad4 activity is observed in about one third of colorectal cancer (CRC) metastases [84] and claudin-1 is an integral tight junction protein frequently over-expressed in CRC carcinomas and metastases [125]. Claudin-1 also has been shown to contribute to the metastatic transformation of CRC cells [125], raising the hypothesis that Smad4 acts as a tumor suppressor by inhibiting claudin-1 expression in CRC. In

the second part of my study, inverse Smad4 and claudin-1 expression was observed in several metastatic CRC tissues and cell lines. Smad4 expression resulted in specific downregulation of claudin-1 protein in two Smad4-deficient, claudin-1-positive colorectal carcinoma cell lines HT29 and SW480. Promoter studies also show suppressed transcription of claudin-1 by Smad4 expression.

Previous findings by others suggest that Smad4 expression in Smad4-deficient SW480 colon carcinoma cells did not restore cell growth inhibitory response to TGF-β presumably due to low levels of type II TGF-β receptor [85]. These data suggest that Smad4 may elicit tumor suppressive responses independently of TGF-β signaling. Results from my studies show that in SW480 cells, ectopic Smad4 protein expression was not sufficient to restore TGF-β-induced promoter activation of the p3TP-Lux, a luciferase reporter construct highly responsive to TGF-β [31]. Moreover, treatment with the selective TGF-β receptor kinase inhibitor, LY364947, failed to prevent the inhibition of claudin-1 in Smad4 expressing SW480 cells. In HT29 cells, although Smad4 re-expression restored autocrine and paracrine TGF-β signaling in terms of promoter activation of the p3TP-Lux, LY364947 did not prevent the Smad4 effect on claudin-1. We thus conclude that Smad4 inhibition of claudin-1 expression is independent of TGF-β signaling in colorectal cancer cells.

CHAPTER II

MATERIALS AND METHODS

Cell cultures and reagents

MDA-MB-231, RIE, HCT15, HCT116, SW480, SW620, HT29 cells from American Type Culture Collection (Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) (MDA-MB-231) or RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in the presence of 5% CO_2 at 37 °C. TGF-β was purchased from R & D Systems (Minneapolis, MN, USA). The pharmacological TGF-β type I and II receptor kinase inhibitor (LY364947) [74] was provided by Eli Lily (Indianapolis, IN, USA). Stable SW480 clones were generated by transfection with a Smad4 expression vector followed by selection in 1 mg/ml G418 media.

Plasmid construction

To construct the luciferase reporter plasmid under the control of the claudin-1 promoter, the -1160 to +160 upstream area of the human *claudin-1* gene was amplified by polymerase chain reaction (PCR) using human genomic DNA with a set of forward and reverse primers containing MluI and XhoI restriction enzyme sites, respectively. The sequences of the primers are 5'-TCGACGCGTACTCGCACCACACACAAAAA-3' (forward primer with MluI site underlined) and 5'-CCGCTCGAGGTGCAGAAGGCGGAGAGTT-3' (reverse primer with XhoI site underlined). PCR was carried out in a 50 μl mixture containing 2.5 U of Taq DNA polymerase, 2 mM $MgCl_2$, 250 μM dNTP (each), 0.4 μM of primers (each), and 100 ng human

genomic DNA. Following an initial denaturation step at 95°C for 5 minutes, the PCR mixture was subjected to 35 cycles of denaturation, annealing, and amplification at 94°C for 40 seconds, 58°C for 30 seconds, and 72°C for 40 seconds, respectively, and a final extension at 72°C for 5 minutes. The PCR product was size-fractioned by 1% (w/v) agarose gel electrophoresis, stained with ethidium bromide, excised from the gel, phenol/chloroform purified, and dissolved in water. After MluI and XhoI enzyme digestion, the PCR product was purified with phenol/chloroform then cloned into the promoter-less luciferase vector, pGL3-Basic (Promega, Madison, WI, USA) and the sequence of the PCR product was confirmed by sequencing analyses.

Protein lysate preparation and immunoblotting analysis

To harvest protein lysates, cells on plates were rinsed with cold phosphate-buffered saline (PBS) and lysed in RIPA buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate, and 1 mM sodium fluoride] for 20 minutes on ice. Lysates were sonicated and then clarified by centrifugation at 15,000 x g for 15 minutes at 4°C. Protein concentrations of lysates were determined by the Bradford Assay (BioRad Laboratory, Inc., Hercules, CA, USA). Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. Membranes were blocked with 5% milk in PBS-T [0.1% Tween 20 (v/v) in PBS] for 1 hour at RT and then probed with primary antibodies in 5% milk PBS-T overnight at 4°C. After several washes with PBS-T, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies in PBS-T for 1 hour at RT and washed again with PBS-T. Immunoreactive bands were visualized by chemiluminescence reaction using

ECL reagents (Amersham Life Science, Arlington Heights, IL, USA) followed by exposure of the membranes to XAR5 films (Kodak, Rochester, NY, USA). To detect secreted uPA, conditioned media were collected, centrifuged at 15,000 x g for 5 minutes to remove cell debris, and then immunoblotted under non-reducing conditions. The volumes of conditioned media loaded on gels were normalized to the protein concentrations of cell lysates. The fibronectin antibody was purchased from Transduction Laboratories, Inc. (San Diego, CA, USA). The Smad2 and phospho-Smad2 antibodies were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). The uPA antibody was obtained from American Diagnostica, Inc. (Greenwich, CT, USA). The PARP and Rho GDI antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The actin and FLAG antibodies were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). The claudin-1, -4, and -7 antibodies were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). The Smad4 antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The actin antibody was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). The E-cadherin and β-catenin antibodies were purchased from BD Biosciences (San Jose, CA, USA).

Preparation of plasma membrane fractions

Cells were collected in buffer containing 0.15 M NaCl, 20 mM HEPES, 2 mM $CaCl_2$, 100 μg/ml leupeptin, 2.5 mg/ml pepstatin A, and 1 mM phenylmethylsulphonylfluoride (pH 8.0) and lysed by freeze/thaw (liquid N_2/42°C) cycles. Nuclei were isolated from the suspension of lysed cells by centrifugation at 7,000 x g for 20 minutes at 4°C, washed three times, and re-suspended in RIPA buffer. The nucleus-free supernatant was spun at 100,000 x g for 1 hr at 4°C

to separate cytoplasm and membrane. Membrane pellets were subsequently washed 3 times with 3 ml of the cell resuspension buffer and dissolved in RIPA buffer.

Transient transfection and luciferase reporter assay

On the day before transfection, cells were seeded on 12-well plates in triplicate and allowed to grow overnight to 50-70% confluence. The following day, cells were co-transfected with a firefly luciferase reporter construct and a reference construct containing *Renilla reniformis* luciferase, phRL-TK, (Promega, Madison, WI, USA) using Lipofectamine Plus reagents (Invitrogen, Carlsbad, CA, USA). Four hours after transfection, cells were returned to regular culture media for 48 hours then lysed. Firefly and *R. reniformis* luciferase activities were measured using the Dual Luciferase Reporter Assay System kit (Promega, Madison, WI, USA) in an Optocomp II Luminometer (MGM Instruments, Inc., Hamden, CT, USA). Firefly luciferase activity was normalized to *R. reniformis* luciferase activity and plotted as mean ± SD from three independent experiments. The phuPA-Luc promoter-reporter containing the -2345 to +30 region of the human uPA promoter was kindly provided by Dr. Shuji, Kojima [131]. The p3TP-Lux reporter was a generous gift from Dr. Joan Massague [132].

Matrigel invasion assay

A modified Boyden chamber assay was performed using Transwells (8 μm pore size, 6.5 mm in diameter) from Costar (Cambridge, MA, USA) and Matrigel (BD Biosciences, Bedford, MA, USA). Transwell inserts were coated with 80 μg of Matrigel each and left for 3 hours at 37°C before use. Cells were trypsinized, washed with PBS, re-suspended in 0.2% BSA serum-free medium, seeded onto Transwell inserts (50,000 cells/insert), and grown in the presence of

10% FBS in the medium in the lower compartment. After 16 hours, cells remaining inside the inserts were removed with Q-tips and the cells that had traversed to the reverse side of the inserts were rinsed with PBS, fixed in 4% formaldehyde for 30 minutes at RT, and stained with 1% crystal violet for 1 hour to overnight at RT. Cells were counted under a light microscope (at 400 x power) and the number of invasive cell shown as the average of five areas on each insert. Each invasion assay was performed in triplicate and repeated three times.

Over-expression of Smad4 by Adenoviral infection

To amplify adenoviruses, the packaging 293T cells cultured in 5% serum DMEM at 80% confluence on P100 plates were infected with adenoviruses in 1 ml fresh 5% serum medium with rocking. After 3 hours, 9 ml of 5% serum medium was added in each plate without removing infection medium. Infected cells were allowed to grow for 3 days before being trypsinized and collected in 1 ml of 5% FBS medium, and subjected to three freeze/thaw cycles at -20°C/37°C. Adenovirus-containing supernatant was obtained from the cell suspension after centrifugation at 15,000 x g for 20 minutes at 4°C and stored at -80°C. The relative values of multiplicity of infection (MOI) of adenoviral suspension were determined by examining the cytopathic effect (CEP) in 293T. Adenovirus with an MOI of 10-20 causes a total CPE in confluent 293T cells 3 days post infection. To perform adenoviral-mediated overexpression of Smad4, 70-80% confluent cells were washed once with PBS and infected with adenovirus at the indicated MOIs for three hours, then returned to regular serum media. Cell lysates were harvested 48-72 hours post infection.

Cell proliferation (MTT) assay

Cells were seeded in 96-well plates (50,000/well) and the relative viable cell numbers were determined by MTT assay using the CellTiter 96 Non-radioactive Cell Proliferation Assay kit (Promega, Madison, WI, USA), following the manufacturer's protocol. MTT hydrolysis was determined by measuring the absorbance at 570 nm using a plate reader.

MMP zymography

Serum-free conditioned medium was mixed with 2 x sample buffer (0.5 M Tris-HCl pH 6.8, 5% SDS, 20% glycerol and 1% bromophenol blue) and subject to SDS-PAGE using 10% SDS-gelatin (1 mg/ml final concentration) gels under non-reducing conditions. After electrophoresis, gels were soaked in washing buffer (50 mM Tris-HCl pH 7.5, 0.1 M NaCl, and 2.5% Triton X-100) for 1 hour at RT to remove SDS and then in reaction buffer (50 mM Tris HCl pH 7.5 and 5 mM $CaCl_2$ pH 8.0) overnight at 37°C. Subsequently, gels were stained in staining buffer (0.15% Coomassie blue R250 in 10% acetic acid and 30% methanol) and de-stained in the same staining solution without Coomassie blue R250. Clear bands of pro- and active MMP-9 (92 kDa and 84 kDa, respectively) were observed against the blue background of stained gels.

RNA isolation and Northern blot analysis

Total RNA isolated with the Trizol reagent was resolved on formaldehyde agarose gels, transferred, and immobilized onto Hybond-N nylon membranes (Amersham Life Science, Arlington Heights, IL, USA). Blots were blocked in ULTRAHyb buffer (Ambion, Austin, TX, USA) for 3 hours at 65°C and probed with ^{32}P-labeled antisense riboprobes (5 x 10^5 cpm/ml) in

ULTRAHyb buffer overnight at 68°C. After several washes with low stringency (2 x SSC/0.1% SDS) and high stringency (0.1 x SSC/0.1% SDS) buffers at 68°C, images were acquired by autoradiography using a phosphorImager. To prepare riboprobes, plasmids containing cDNAs were linearized, purified, and then subject to *in vitro* transcription using the MAXIscript kit (Ambion, Austin, TX, USA) in the presence of 50 μCi of [α-^{32}P] UTP (800 Ci/ mmol) for 1 hour at 37°C. Unincorporated nucleotides were removed using NucAway spin columns (Ambion, Austin, TX, USA).

Nuclear run-on assay

Cell were collected, washed twice with PBS, and then re-suspended in lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 1 mM DTT). NP-40 was added to a final concentration of 0.2 to 0.5%, depending on the cell type. After a 5-minute incubation on ice, nuclei were pelleted at 500 x g for 5 minutes, washed once with nuclear freezing buffer (50 mM Tris-HCl pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 1 mM DTT), and re-suspended in fresh nuclear freezing buffer. *In vitro* run-on transcription was performed using 2 x 10^7 nuclei in 150 μl reaction buffer [5 mM Tris-HCl pH 8.0, 2.5 mM $MgCl_2$, 150 mM KCl, 1 mM of ATP, CTP, GTP, 150 μCi of [α-^{32}P] UTP (800 Ci/ mmol), 80 units of RNasin, and 2.5 mM DTT] during a 30-minute incubation at 30°C. Transcription was terminated by adding 350 μl of deoxyribonuclease I solution (20 mM Tris-HCl pH 7.4, 10 mM $CaCl_2$, and 300 units of RNase-free DNase I) and incubated for 30 minutes at 37°C. Next, proteins were digested by adding 50 μl of proteinase K solution (1% SDS, 5 mM EDTA, 1 mM Tris-HCl pH 7.4, and 300 μg/ml proteinase K) and incubated for 30 minutes at 50°C. The ^{32}P-labelled RNAs were phenol/chloroform purified and precipitated in 10% trichloroacetic acid plus 20 μg yeast tRNA.

After centrifugation at 15,000 x g for 1 hour, the RNAs were re-suspended in RNase-free H_2O, denatured for 10 minutes at 65°C, and then chilled on ice. The radiolabeled RNAs were hybridized to cDNAs pre-immobilized on membranes in hybridization buffer (50% formamide, 5 x SSC, 5 mM EDTA, 5 x Denhardt's solution, 0.1% SDS, and 100 ug/ml denatured salmon sperm DNA) for 48 hours at 42°C. Next, the membranes were washed several times with 2 x SSC (1 x SSC = 0.15 M NaCl and 12.5 mM Na-citrate pH 7) and then with 2 x SSC plus 10 μg/ml RNase A for another 30 minutes or not depending on intensity of background. Signals were acquired and quantified with a PhosphorImager. To immobilize cDNAs on nitrocellulose membranes, 1 μg linearized plasmid was denatured in 0.2 M NaOH for 30 minutes at RT and then neutralized with 10 volumes of 6 x SSC. The DNAs were applied to nitrocellulose membranes using a slot blot apparatus and immobilized by UV crosslinking.

RNA Interference

To perform Smad4 silencing, 70% confluent cells were transfected with 50 or 200 pM of a pool of four Smad 4 or scrambled siRNAs (Dharmacon, Lafayette, CO USA) using Oligofectamine (Invitrogen, Carlsbad, CA, USA) according to manufacturer's guideline. Conditioned media and protein lysates were harvested 3 days post transfection and subjected to immunoblotting.

Immunofluorescence study

Cells grown in multi-chamber slides were fixed in 4% paraformaldehyde/PBS for 30 minutes at RT and permeabilized in 0.1% Triton for 30 minutes at RT. Cells were then blocked in 10% normal goat or donkey serum for 1 hour at RT followed by incubation with primary

antibody at the indicated dilutions (1:200 for claudin-1, Smad2, Smad1/5/8, and pSmad1/5/8 antibodies; 1:400 for Smad4 and E-cadherin antibodies) in 2.5% normal goat or donkey serum overnight at 4°C. After several washes with PBS, cells were incubated with FITC- or Rhodamine-conjugated secondary antibodies (Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA) at a 1:400 dilution in 2.5% normal goat or donkey serum for 1 hour at RT. After PBS washes, cells were mounted with a DAPI-containing mounting medium and viewed under a fluorescence microscope.

CHAPTER III

AUTOCRINE TGF-β REGULATES UROKINASE PLASMINOGE ACTIVATOR AND INVASIVENESS IN BREAST CANCER

Abstract

Metastasis is a primary cause of mortality due to cancer. Early metastatic growth involves both a remodeling of the extracellular matrix (ECM) surrounding tumors and invasion of tumors across the basement membrane. Upregulation of ECM degrading proteases contributes to invasion of the basement membrane in tissues. Autocrine TGF-β signaling may play an important role in cancer cell metastasis. TGF-β regulation of tumor cell invasiveness has been linked with expression of uPA; however, little is known about the molecular mechanisms by which autocrine TGF-β regulates uPA or whether this regulation is causally linked with the invasiveness of tumor cells. In the present study, inhibition of autocrine TGF-β signaling using a pharmacological inhibitor of TGF-β receptors (LY-364947) or with a dominant-negative TGF-β type II receptor decreased uPA secretion and tumor cell invasiveness. Inhibition of uPA proteolytic activity with a blocking antibody decreased cell invasion to the same extent as did inhibition of autocrine TGF-β signaling. Inhibition of autocrine TGF-β signaling decreased uPA protein secretion, but not intracellular uPA protein or mRNA levels. In contrast, exogenous TGF-β increased uPA mRNA expression through RNA stabilization. Blockade of TGF-β signaling with LY36497 efficiently inhibited Smad2 phosphorylation induced by TGF-β treatment, but did not affect the basal activation status of ERK, p38, Akt, and Src proteins, other potential effectors downstream of TGF-β signaling. Further, suppression of Smad4 expression by RNA interference decreased uPA secretion. We conclude that autocrine TGF-β regulates

uPA secretion via a Smad-dependent pathway, thereby promoting tumor cell invasion, whereas exposure to higher concentrations of exogenous TGF-β results in increased uPA mRNA stability leading to both increased production and release of uPA and further increases in cell invasiveness.

Introduction

Malignant tumors are characterized by their ability to metastasize to distant organs. The initial steps of metastasis involve invasive growth of tumors across the basement membrane and migration through the extracellular matrix (ECM). As the enzymatic degradation of the basement membrane and ECM barriers requires a number of ECM-degrading proteases [38, 133] and is a critical early event in metastasis, invasiveness may be modulated by the expression of ECM-degrading proteases in tumor cells in response to autonomous and microenvironmental signals. Among the increasing number of ECM-degrading proteases implicated in metastasis, considerable attention has been focused on the family of matrix metalloproteinases (MMPs) and the plasminogen activator system. One of the regulators of these ECM-degrading proteases is transforming growth factor-β (TGF-β).

TGF-β is a multifunctional cytokine that regulates cell proliferation, differentiation, plasticity, and migration in a context-dependent manner (reviewed in references [68, 134]). TGF-β transduces signaling through a transmembrane type II receptor (TβRII), a constitutively active serine/threonine kinase receptor [135]. Upon ligand binding, the TβRII recruits and transphosphorylates intracellular TGF-β type I receptor (TβRI), thereby stimulating TβRI serine/threonine kinase activity [136]. The TβRI then activates the downstream effectors, Smad2 and Smad3 by phosphorylation. The activated Smad proteins form complexes with the common Smad mediator, Smad4 and then translocate to the nucleus, where the Smad complexes regulate

transcription of numerous TGF-β target genes. Other signaling pathways may be activated by TGF-β depending on cell context. These pathways include the extracellular signal-regulated kinases (ERK1/2) [17, 137], mitogen-activated protein kinase (p38) [138, 139], Src [111], and the phosphatidylinositol 3-kinase (PI3K) [62] pathways. The precise molecular mechanisms of regulation of these pathways for TGF-β signaling and the physiological and pathological roles of TGF-β in normal tissues and cancer have not been completely defined.

Important roles for autocrine TGF-β in tumor progression and metastatic behavior have been supported by previous observations. For instance, disruption of autocrine TGF-β signaling by a dominant-negative type II receptor (DNIIR) inhibited the invasive and metastatic potential of mammary and colon carcinoma cells [49]. This was attributed to prevention of autocrine TGF-β-induced epithelial-to-mesenchymal transition (EMT), a process believed to promote tumor cell migration and invasion [62]. Inhibition of autocrine TGF-β signaling by over-expression of a soluble TGF-β type III receptor antagonized TGF-β activity and resulted in inhibition of tumor cell proliferation and induction of apoptosis [40].

The urokinase plasminogen activator (uPA) is a serine protease capable of initiating cascades of activation of ECM-degrading enzymes [64] and eliciting intracellular signaling through receptor binding. Numerous studies have linked uPA to malignant phenotype of tumors [140, 141]; its role in invasion and metastasis has also been demonstrated in cell culture and animal models [51, 142]. Clinically, elevated uPA expression in tumors is associated with tumor aggressiveness and poor outcome in patients [65, 66]. The metastatic MDA-MB-231 breast cancer cells secrete active TGF-β [143, 144] and are TGF-β responsive [145]. These cells also express both the matrix metalloproteinases-9 (MMP-9) and uPA [51, 146, 147]. We hypothesized that autocrine TGF-β may function as a tumor promoter by regulating MMP-9 or

uPA activity in MDA-MB-231 cells. The present study provides evidence that autocrine TGF-β regulates both cell invasiveness and uPA secretion. Inhibition of uPA activity is sufficient to suppress tumor cell invasion to the same extent as inhibition of autocrine TGF-β signaling, suggesting that autocrine TGF-β stimulation of invasiveness occurs via its regulation of uPA release. The Smad pathway is required to regulate uPA levels as silencing of Smad4 results in inhibition of uPA secretion. Interestingly, while autocrine TGF-β regulates uPA production through protein secretion, exogenous TGF-β further increases uPA expression, in part, through RNA stabilization. Finally, this work demonstrates that a pharmacological kinase inhibitor of TGF-β receptors inhibits both uPA secretion and tumor cell invasiveness, thereby providing evidence for the potential efficacy of targeting TGF-β signaling for therapeutic intervention in cancer, and suggests that uPA expression or secretion may be an important mediator of such effects.

Results

Autocrine TGF-β signaling contributes to MDA-MB-231 cell invasion

The metastatic MDA-MB-231 breast cancer cells secrete active TGF-β [143, 144] and are TGF-β responsive [145]. The importance of autocrine TGF-β signaling in regulation of MDA-MB-231 cell invasiveness was assessed by Matrigel invasion assays following abrogation of autocrine TGF-β signaling using the LY364947 compound [74], a kinase inhibitor of both TGF-β type I and II receptor (TβRI and TβRII) by functioning as a potent ATP competitive inhibitor.

The inhibitory effect of LY364947 on TGF-β signaling was first validated by examining Smad2 phosphorylation. Basal levels of Smad2 phosphorylation were undetectable by

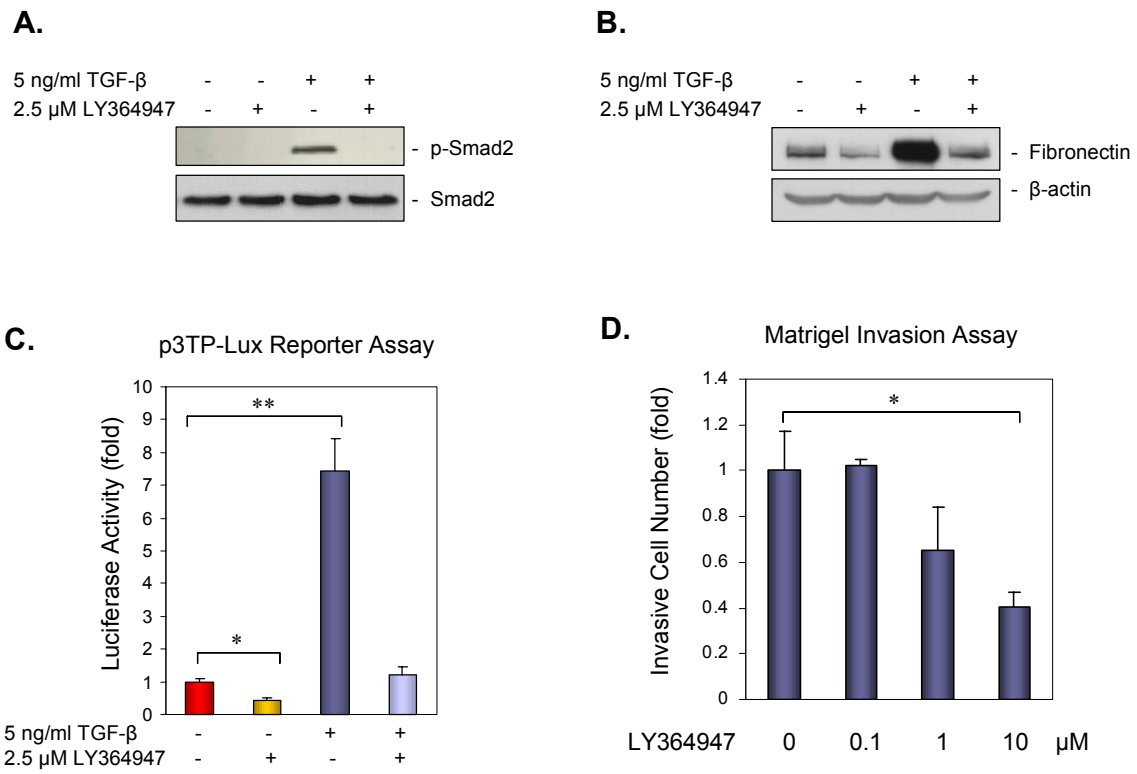


Figure 7. The TGF-β receptor kinase inhibitor (LY364947) inhibited TGF-β signaling and MDA-MB-231 cell invasion. (A) LY364947 inhibition of Smad2 phosphorylation induced by TGF-β. Cells were treated with vehicle, LY364947, TGF-β or in combination as indicated. Forty five minutes later, cell lysates were collected and subjected to immunoblotting for total and phospho-Smad2. (B) LY364947 inhibition of basal and TGF-β-induced fibronectin expression. Cells were treated with vehicle, LY364947, TGF-β, or in combination as indicated. Twenty four hours later, lysates were collected and subjected to immunoblotting for fibronectin and β-actin. (C) LY364947 inhibition of basal and TGF-β-induced promoter activation. Cells were transfected with the p3TP-Lux luciferase reporter construct as described in the "Materials and Methods" and then treated with vehicle, LY364947, TGF-β, or in combination as indicated. Luciferase activity was determined 48 hours after treatments. (D) LY364947 inhibition of MDA-MB-231 cell invasion. Cells were treated with LY364947 at the indicated concentrations overnight and then plated in Matrigel-coated Transwells in the presence of vehicle or increasing concentrations of LY364947 as indicated. Sixteen hours later, cells on the reverse side of each Transwell membrane were stained and counted. The data in (C) and (D) are expressed as fold changes relative to controls. Results represent the average from three independent experiments ± SD. * $P < 0.05$ and ** $P < 0.003$ are derived from one-way ANOVA with a Bonferroni correction.

immunoblotting. However, LY364947 abolished Smad2 phosphorylation induced by exogenous TGF-β without altering total Smad2 protein levels (Fig. 7A). Expression of fibronectin is induced by TGF-β through a Smad-independent pathway [148]. LY364947 also decreased both basal and exogenous TGF-β-induced fibronectin expression (Fig. 7B). In addition, we evaluated the effect of LY364947 on TGF-β-induced promoter activation by reporter assays using p3TP-Lux, a luciferase reporter construct highly responsive to TGF-β [132] and observed that LY364947 significantly inhibited both basal and exogenous TGF-β-induced promoter activation (Fig. 7C). To investigate whether autocrine TGF-β has a role in regulation of MDA-MB-231 invasiveness, Matrigel invasion assays were performed in the presence or absence of LY364947 treatment. LY364947 inhibited cell invasion in a dose-dependent manner (Fig. 7D). These data suggest that autocrine TGF-β plays a role in basal rates of MDA-MB-231 cell invasiveness.

Expression of a dominant-negative TGF-β type II receptor suppresses autocrine TGF-β signaling and cell invasion

To substantiate the results obtained using the kinase inhibitor of TGF-β receptors, we assessed invasiveness of MDA-MB231 cells following suppression of autocrine TGF-β signaling with a dominant-negative TGF-β type II receptor (TβRII). TβRII is the receptor responsible for ligand binding and for activation of TGF-β type I receptor through its kinase activity. TβRII devoid of the kinase domain (DNIIR) acts as a dominant-negative mutant by competing with wild-type receptors for TGF-β ligands [149]. Expression of FLAG-tagged dominant-negative DNIIR was achieved using an adenoviral vector and was confirmed by immunoblotting for FLAG (Fig. 8A). DNIIR expression was increased with increasing amounts of adenovirus whereas no DNIIR was detected in parental or the β-galactosidase adenovirus-infected cells.

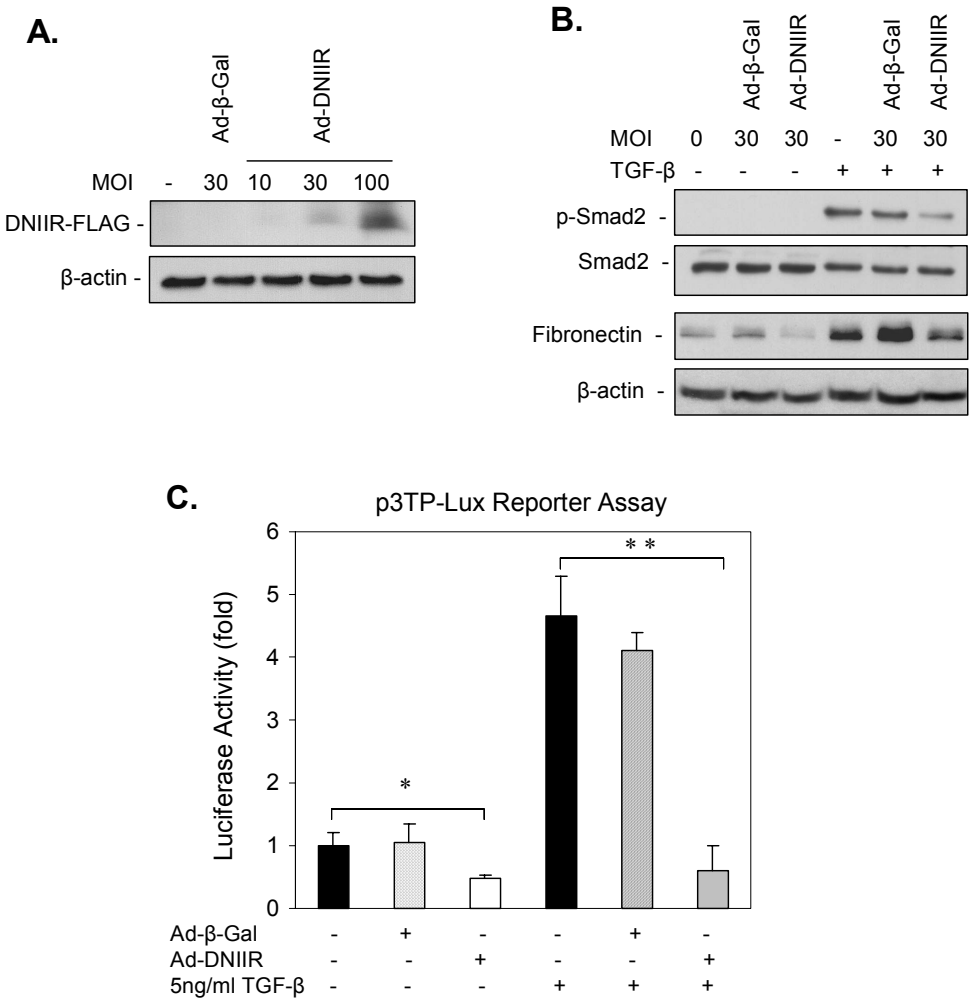


Figure 8. Inhibition of TGF-β signaling by expression of a dominant-negative TGF-β type II receptor (DNIIR). (A) Over-expression of DNIIR-FLAG. Cells were infected with β-galactosidase (Ad-β-Gal) or DNIIR (Ad-DNIIR) adenoviruses at the indicated MOIs for three hours and allowed to grow in fresh media. Forty eight hours later, lysates were prepared and subjected to immunoblotting for FLAG. (B) DNIIR inhibited Smad2 phosphorylation and fibronectin expression induced by TGF-β. Forty eight hours after adenoviral infection, cells were treated with TGF-β for 45 minutes for phospho-Smad2 immunoblotting or for 24 hours for fibronectin immunoblotting. (C) DNIIR attenuated basal and TGF-β-induced promoter activation of p3TP-Lux. Cells were infected with adenoviruses at an MOI of 30. Twenty four hours after adenoviral infection, cells were transiently co-transfected with p3TP-Lux and phRL-TK and allowed to grow for 48 hours in the presence or absence of 5ng/ml TGF-β. Cells were then lysed and the lysates were subjected to reporter assays. *, $P < 0.05$ and **, $P < 0.01$, are derived from one-way ANOVA with a Bonferroni correction; bars, standard deviation.

Expression of DNIIR inhibited TGF-β-stimulated Smad2 activation (Fig. 8B) and decreased both basal and exogenous TGF-β-induced fibronectin expression (Fig. 8B). Expression of DNIIR also suppressed basal and exogenous TGF-β-stimulated p3TP-Lux promoter activation (Fig. 8C), demonstrating the inhibitory effects of DNIIR on TGF-β signaling. As expected, DNIIR expression significantly decreased MDA-MB-231 cell invasion (Fig. 9A). This effect did not result from inhibition of cell proliferation as determined by MTT assay (Fig. 9B). Thus, consistent with the results using the pharmacological inhibitor, inhibition of autocrine TGF-β signaling by the dominant-negative TGF-β type II receptor (DNIIR) downregulated MDA-MB-231 invasiveness.

Disruption of autocrine TGF-β signaling suppresses uPA secretion

The MDA-MB-231 cells secrete uPA [51]. To address whether autocrine TGF-β can modulate uPA expression, uPA levels were determined after blockade of autocrine TGF-β signaling using LY364947. Immunoblotting for uPA was conducted under non-reducing conditions, which detect both uPA and complexes of uPA and its inhibitor, plasminogen activator inhibitor-1 (PAI-1) [150]. The low motility bands with molecular masses around 100 kDa represent uPA/PAI-1 complexes (uPA ~55 kDa and PAI-1 ~52 kDa). Our results show that LY364947 decreased uPA secretion in a dose-dependent manner (Fig. 10A) and that the decreased level of free uPA in the conditioned medium did not result from increased uPA/PAI-1 association and depletion of uPA. MDA-MB-231 cells constitutively express uPA receptor (uPAR) mRNA and exhibit detectable membrane-associated uPA [51]. To determine whether the decreased level of released uPA after LY364947 treatment was the result of increased uPAR expression and increased uPA binding to uPAR, levels of membrane-bound uPA were

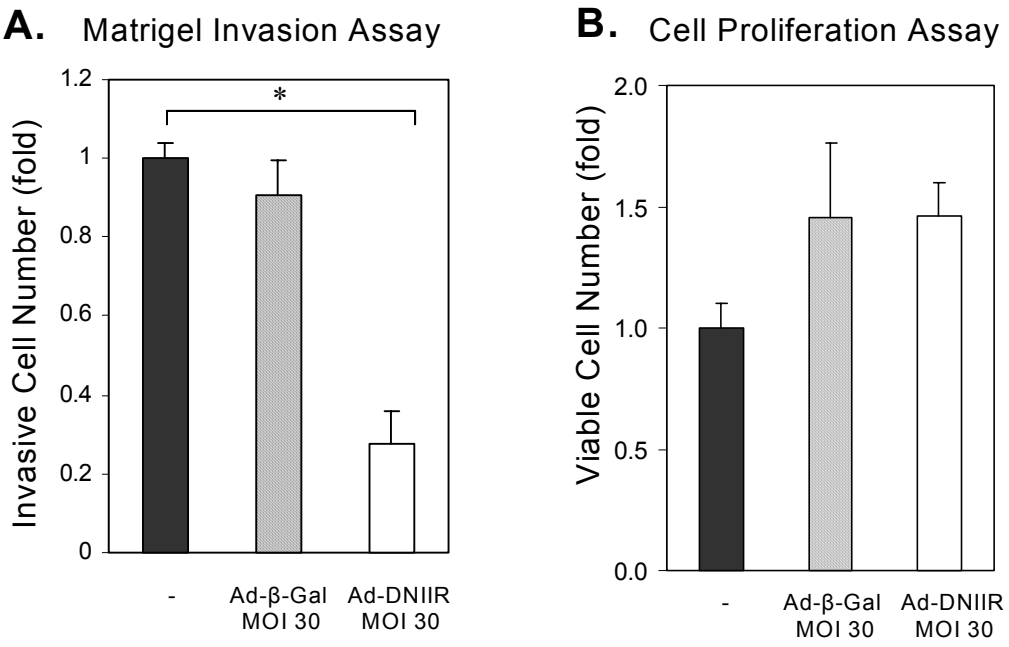


Figure 9. Expression of the dominant-negative TGF-β type II receptor (DNIIR) decreased MDA-MB-231 cell invasion. (A) Cells were infected with adenoviruses at an MOI of 30. Forty eight hours after adenoviral infection, cells were plated in Matrigel-coated Transwells (A) or 96-well plates (B) in triplicate. After 16 hours, invasion (A) or MTT (B) assays were performed as described in "Materials and Methods". *, $P < 0.05$ is derived from one-way ANOVA with a Bonferroni correction; bars, standard deviation.

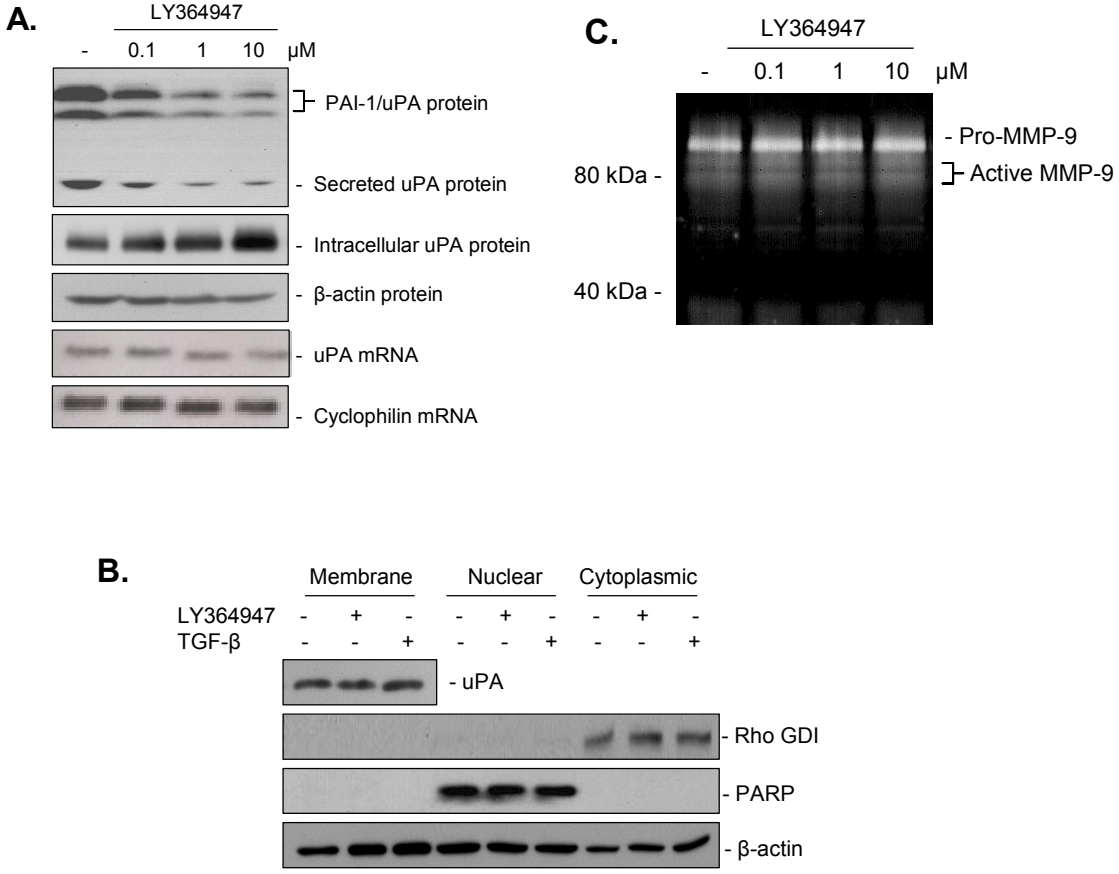


Figure 10. The TGF-β receptor kinase inhibitor, LY364947, decreased uPA but not MMP-9 protein levels in MDA-MB-231 conditioned media. (A) LY364947 decreased uPA secretion. Cells were treated with LY364947 at the indicated concentrations for 24 hours. Conditioned media, cell lysates, and total RNAs were collected and subjected to immunoblotting for uPA and β-actin or Northern blotting for uPA and cyclophilin. (B) The effect of LY364947 on membrane association of uPA proteins. Cells were treated with 5 μM LY364947 or 5 ng/ml TGF-β for 24 hours. Membrane, cytoplasmic, and nuclear fractions were isolated as described in the "Materials and Methods" and subjected to immunoblotting for uPA, PARP (nuclear marker), Rho GDI (cytoplasmic marker), and β-actin. (C) The effect of LY364947 on MMP-9 protein levels and activity. Cells were treated with LY364947 at the indicated concentrations for 24 hours. Condition media were collected and subjected to gelatin zymography.

determined after LY364947 treatment. The purity of the membrane fractions was confirmed by the absence of nuclear and cytoplasmic proteins, PARP (polyadenosine diphosphate ribose polymerase) and Rho GDI, respectively (Fig. 10B). Our results show that neither exogenous TGF-β treatment nor LY364947 treatment altered membrane uPA levels (Fig. 10B). Inhibition of autocrine TGF-β signaling by expression of DNIIR also suppressed uPA secretion in a dose-dependent fashion (Fig. 11). In contrast, β-galactosidase adenovirus infection did not alter uPA secretion, further supporting that inhibition of autocrine TGF-β signaling suppresses uPA secretion. Of note, expression of DNIIR was not detectable at an MOI of 10 by immunoblotting (Fig. 8A); however this low level of DNIIR expression was sufficient to decrease uPA secretion (Fig. 11). These data demonstrate that disruption of autocrine TGF-β signaling inhibited uPA secretion.

Steady-state levels of uPA mRNA did not change with either LY364947 treatment or DNIIR expression (Fig. 10A and 11). Further, while uPA release into the medium was decreased, the intracellular uPA were increased after blockade of autocrine TGF-β signaling (Fig. 10A and 11), suggesting that autocrine TGF-β stimulates uPA secretion and that inhibition of autocrine TGF-β signaling reduces the level of secretion without impairing uPA production, thus leading to intracellular accumulation of uPA.

Disruption of autocrine TGF-β signaling does not affect MMP-9 protein levels and activity

MDA-MB-231 cells express the MMP-9 protein [51] that is particularly important among at least 19 MMP proteins identified to date in tumor invasion and metastasis due to its ability to degrade the basement membrane component, type IV collagen. We determined the effect of LY364947 on MMP-9 protein levels and activity. Gelatin zymography shows no change in

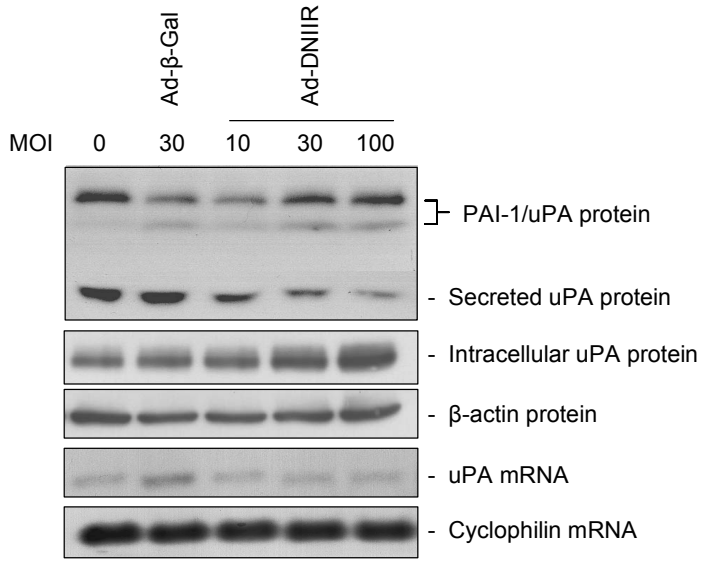


Figure 11. Expression of the dominant-negative TGF-β type II receptor (DNIIR) decreased uPA secretion. Cells were infected with β-galactosidase (Ad-β-Gal) or DNIIR (Ad-DNIIR) adenoviruses at the indicated MOIs. Forty eight hours after infection, cells were cultured in fresh medium and allowed to grow for 16 hours. Conditioned media, protein lysates, and total RNA were harvested and subjected to immunoblotting for uPA and β-actin or Northern blotting for uPA and cyclophilin.

either MMP-9 (92 kDa) protein levels or MMP-9 activity after LY364947 treatment (Fig. 10C), indicating that autocrine TGF-β does not regulate MMP-9 activity or protein expression in the MDA-MB-231 cells. Consistent with previous reports [51], we did not detected MMP-2 (72 kDa) expression in MDA-MB-231 cells.

Inhibition of uPA activity impairs MDA-MB-231 cell invasion

Inhibition of autocrine TGF-β signaling resulted in decreased invasiveness and uPA secretion as described above. We next investigated a relationship between uPA activity and MDA-MB-231 cell invasiveness. Inhibition of uPA activity using an anti-catalytic uPA blocking antibody attenuated cell invasion by ~70% as compared with no treatment or IgG treatment in Matrigel invasion assays (Fig. 12A), indicating a correlation between of uPA activity and MDA-MB-231 cell invasiveness. Parallel MTT assays suggest that the decreased cell invasion was not due to alterations in cell proliferation (Fig. 12B). Thus, uPA activity contributes to cell invasiveness and uPA appears to be the important mediator of invasiveness due to autocrine TGF-β signaling.

Smad4 RNA interference decreases uPA secretion

TGF-β receptors transduce signals through both the Smad pathway and the Smad-independent pathways such as the extracellular signal-regulated kinases (ERK1/2) [17, 137], the p38 mitogen-activated protein kinase [138, 139], the phosphatidylinositol 3-kinase (PI3K) [62], and the Src [111] pathways. Whether autocrine TGF-β regulates those Smad-independent pathways was evaluated by immunoblotting. LY364947 treatment (up to 10 μM) did not significant alter activation status of ERK1/2, p38, Akt, and Src proteins (Fig. 13A). In contrast,

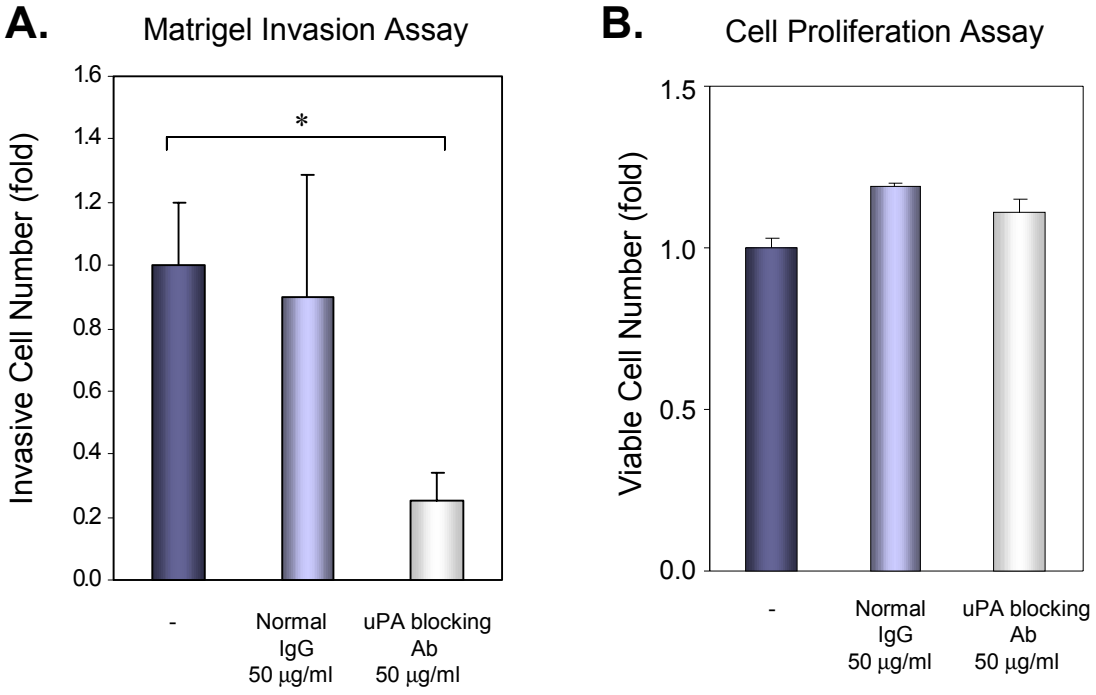


Figure 12. Inhibition of uPA activity decreased MDA-MB-231 cell invasion. Cells were plated and grown in Matrigel-coated Transwells (A) or 96-well plates (B) in the presence or absence of 50 μg/ml of a normal IgG or an uPA activity blocking antibody. Sixteen hours later, invasive and viable cell numbers were determined by Matrigel invasion and MTT assays, respectively, as described in the "Materials and Methods". * $P < 0.03$ is derived from one-way ANOVA with a Bonferroni correction; bars, standard deviation.

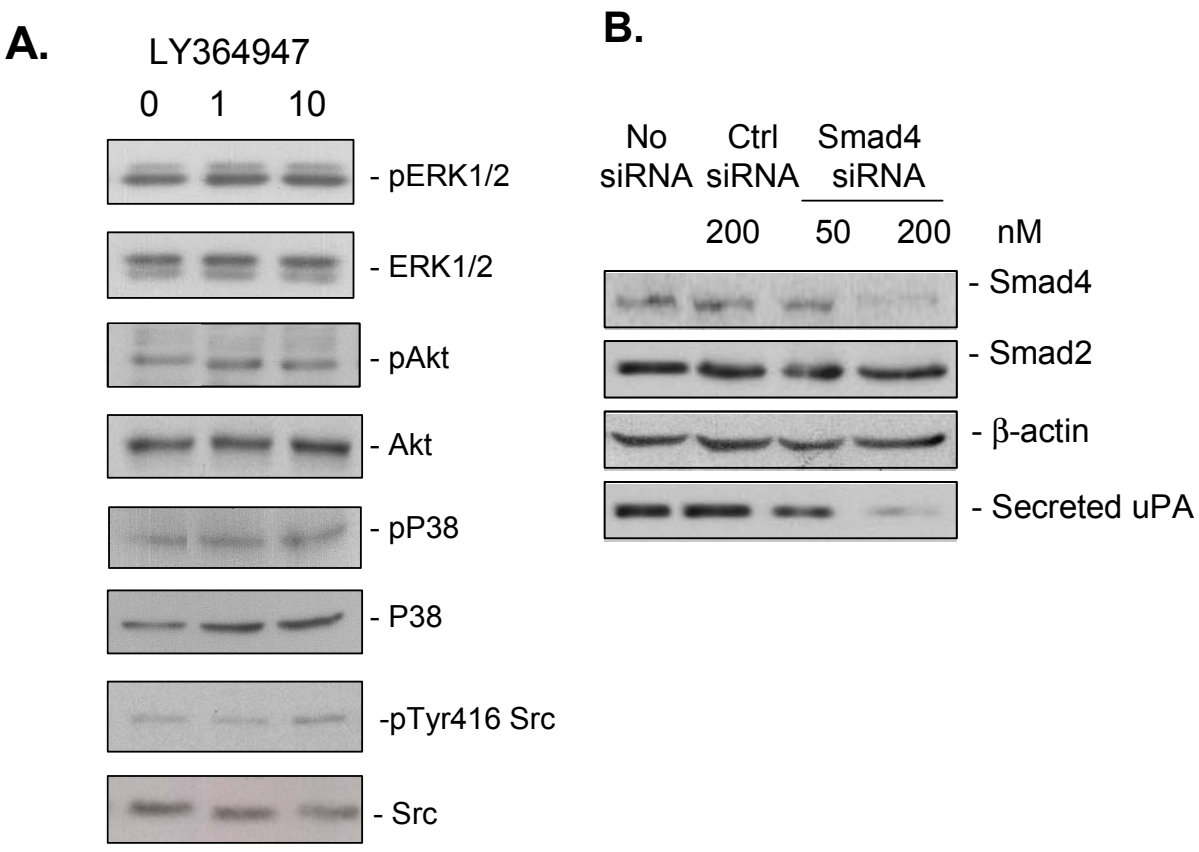


Figure 13. Smad4 silencing decreased uPA secretion. (A). Inhibition of autocrine TGF-β signaling by LY364947 did not alter activation status of ERK1/2, Akt, p38, and Src proteins. Cells were treated with 0, 1, and 10 μM LY364947 for 24 hours and then lysed. Immunoblotting was performed using antibodies against total and active forms of ERK1/2, Akt, p38, and Src proteins. (B) Smad4 silencing decreased Smad4 protein expression and uPA secretion. Cells were transiently transfected with a Smad4 or a scrambled control siRNA at the indicated concentrations for 24 hours. Cells were then allowed to grow in fresh medium for another 24 hours. Conditioned media and lysates were harvested and subjected to immunoblotting for uPA, Smad2, Smad4, and β-actin.

as little as 0.1 μM of LY364947 suppressed uPA secretion (Fig. 10A), implicating that these pathways are probably not involved in autocrine TGF-β regulation of uPA secretion. We hypothesized that the Smad pathway mediates the autocrine TGF-β effect on uPA secretion. In this case, downregulation of the common Smad mediator, Smad4, should suppress uPA release that results from autocrine TGF-β signaling. Smad4 silencing was achieved using a mixture of four Smad4 siRNAs. Immunoblotting results confirmed decreased Smad4 expression in Smad4 siRNA-transfected cells but not in parental or scrambled control siRNA-transfected cells (Fig. 13B). Smad2 protein expression was unaffected by the Smad4 siRNA transfection, further confirming specific silencing of Smad4 protein expression by the Smad4 siRNAs. Interestingly, uPA secretion was also decreased by inhibition of Smad4 protein expression (Fig. 13B), indicating that the Smad pathway is required for the regulation of uPA secretion by autocrine TGF-β.

Exogenous TGF-β increases uPA mRNA levels through RNA stabilization

Exogenous TGF-β was previously shown to stimulate uPA expression and increased MDA-MB-231 cell invasiveness [51]. We further examined uPA regulation by exogenous TGF-β. Unlike autocrine TGF-β, exogenous TGF-β increased both uPA mRNA and protein levels in a dose-dependent manner (Fig. 14A). A time course study shows that uPA mRNA was induced by TGF-β in a time-dependent fashion to near maximal levels by 16 hours after treatment and the level was sustained for at least 48 hours (Fig. 14B). Increased mRNA expression is due to either increased transcription or increased RNA stability. The transcription of uPA in response to exogenous TGF-β was determined by a nuclear run-on assay. Exogenous TGF-β failed to enhance uPA transcription (Fig. 15A and B) but strongly stimulated transcription of PAI-1, a

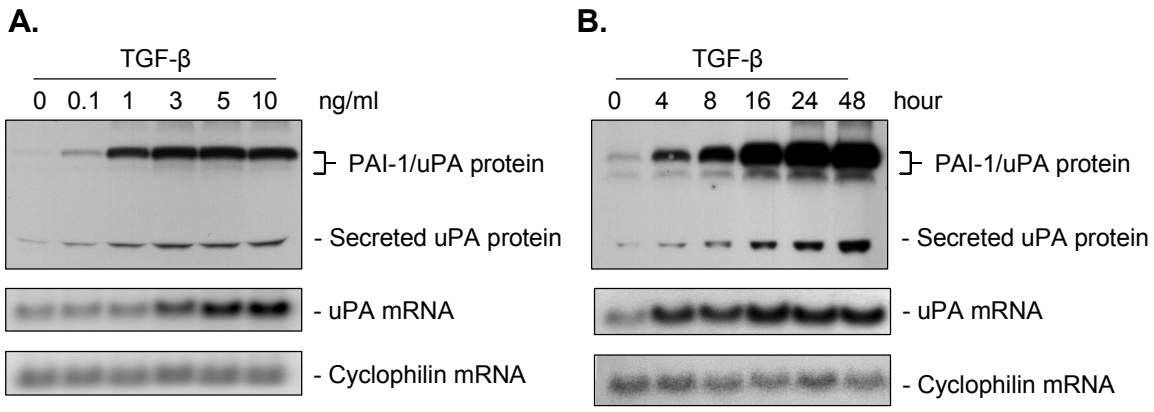


Figure 14. Exogenous TGF-β increased uPA mRNA levels and protein secretion in a dose- and time-dependent manner. Cells were treated with increasing concentrations (0.1 – 10 ng/ml) of TGF-β for 24 hours (A) or 5 ng/ml TGF-β for various intervals (B) as indicated. Conditioned media, lysates and total RNA were harvested and subjected to immunoblotting for uPA or Northern blotting for uPA and cyclophilin, respectively.

TGF-β target gene that has been shown to be transcriptionally activated by TGF-β [151]. Consistent with the nuclear run-on result, transient expression assays using a uPA-promoter-reporter construct containing the nucleotide -2345 to +30 region of the human uPA promoter (phuPA-Luc) show very little changes in uPA promoter activity after TGF-β treatment (Fig. 7C). In contrast, uPA promoter activity was significantly induced by PMA, a known inducer of activation of the human uPA promoter [152] (Fig. 15C). Next, the stability of uPA mRNA in the presence and absence of exogenous TGF-β was determined by examining uPA mRNA levels at various time points after blocking transcription with the RNA polymerase II-specific inhibitor DRB (5, 6- dichloro-1-β-D-ribofuranosyl-benzimidazole). Quantitation of the Northern blotting results shown in Fig. 16A indicates that by 8 hours after DRB treatment, uPA mRNA levels were decreased by 50% in untreated cells (Fig. 16B). In contrast, uPA mRNA levels were only decreased by 25% in TGF-β-treated cells, suggesting that exogenous TGF-β enhances the stability of uPA mRNA. Thus, exogenous TGF-β increased uPA mRNA levels through mRNA stabilization in MDA-MB-231 cells.

Discussion

TGF-β is a potent inhibitor of epithelial cell growth through inhibition of proliferation and induction of apoptosis [35, 153] and is an important tumor suppressor [154]. The tumor suppressor role of TGF-β is evident in that mice heterozygous for deletion of the TGF-β gene with expression of 10% to 30% of TGF-β levels of wild type animals, developed an increased number of chemically-induced tumors than did wild-type littermates [154]. However, escape from the growth inhibitory effects of TGF-β occurs frequently in cancer through numerous mechanisms. TGF-β also displays tumor promoting effects in late-staged tumors. TGF-β has

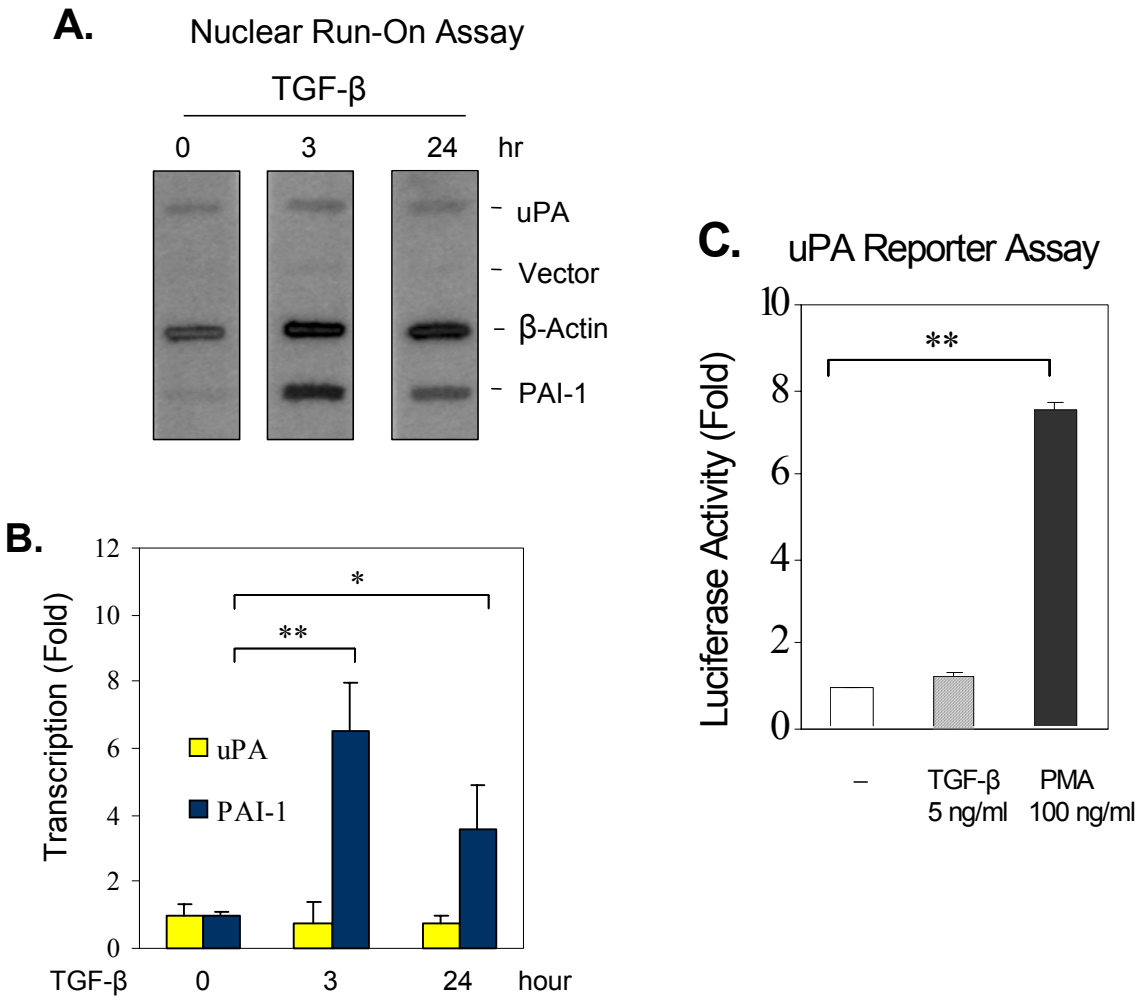


Figure 15. Exogenous TGF-β did not stimulate uPA transcription. (A) After cells were treated with 5ng/ml TGF-β for 0, 3, or 24 hours, nuclei were harvested and subjected to nuclear run-on assays as described in "Materials and Methods". Signals were visualized and quantified by a PhosphorImager and plotted as fold change (B). (C) Exogenous TGF-β did not increase uPA promoter activity. Cells were co-transfected with phuPA-Luc and phRL-TK for 24 hours and then treated with TGF-β or PMA for 48 hours before reporter assays. All data are presented as the mean ± SD. *, $P < 0.03$ and **, $P < 0.01$ are derived from one-way ANOVA with a Bonferroni correction.

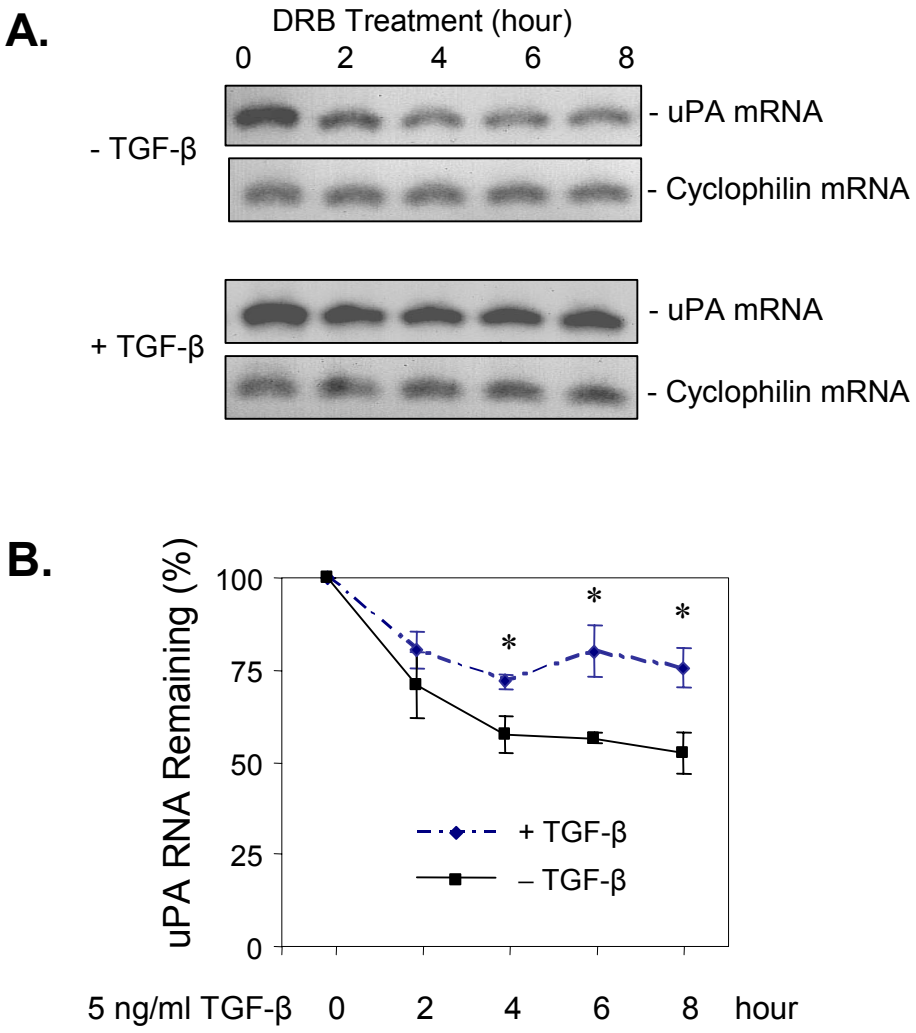


Figure 16. Exogenous TGF-β stabilized uPA mRNA. (A) TGF-β decreased uPA mRNA turnover. Cells were treated with vehicle or 5 ng/ml TGF-β for 16 hours and then with 20 μg/ml of DRB in the presence or absence of TGF-β. Total RNA was isolated at the indicated times after DRB treatment and subjected to Northern blotting for uPA and cyclophilin. (B) Cyclophilin-normalized uPA mRNA levels from (A) were plotted as percentages of the uPA level at the 0 hour time point. *, $P < 0.05$ is derived from t test; bars, standard deviation.

been shown to facilitate tumor progression by inducing EMT [48, 155], ECM degradation [51], and cyclooxygenase-2 expression [156] or by inhibiting anti-tumor immune responses [59].

Disruption of tumor autocrine TGF-β signaling has been found to delay tumor growth and inhibit metastases [157] [49]. However, the molecular mechanisms underlying the tumorigenic effects of autocrine TGF-β remain unclear. The present study demonstrates that autocrine TGF-β regulates both cell invasiveness and uPA secretion. Inhibition of uPA activity decreases tumor cell invasion to the same extent as does the inhibition of autocrine TGF-β signaling. Interestingly, while autocrine TGF-β regulates uPA availability via effects on protein secretion, exogenous TGF-β further increases uPA availability by increasing uPA mRNA through RNA stabilization. Our data suggest two distinct levels of regulation of uPA in response to different magnitudes of stimulation by TGF-β. The amount of active TGF-β in MDA-MB-231 cell conditioned media has been quantitated at ~0.25 ng/ml [63, 143, 144] while at least 10-fold higher concentrations that were required to increase uPA mRNA levels (see Figure 6A).

Stabilization of mRNA involves the binding of RNA proteins to certain cis-elements of mRNAs [158]. Adenylate-uridylate-rich elements (AREs) are important regulatory cis-elements present in the un-translated regions (UTRs) of short-lived mRNAs such as proto-oncogenes, cyclooxygenase-2 [159] and c-fos [160] mRNAs. TGF-β and Ras have been shown to synergistically stabilize the COX-2 mRNA through an ARE in the proximal 3'-untranslated regions (3'-UTRs) [159]. Given that MDA-MB-231 cells possess an activating Ki-Ras mutation [161] and that AREs are present in the 3'-UTR of the uPA mRNA [162], it is possible that TGF-β may cooperate with the active Ras to regulate the stability of uPA mRNA through a similar mechanism, but confirmation of this awaits further investigation.

Cells release uPA as a single-chain zymogen. The low level of intrinsic proteolytic activity of pro-uPA [163] can convert the plasminogen in tumor microenvironments or Matrigel [164] to plasmin, which in turn activates pro-uPA. This pro-uPA activation by plasmin and activation of plasminogen by uPA in a cyclic fashion promotes degradation of the extracellular matrix (ECM) or the basement membrane and facilitates cell invasion. In addition, plasmin can potentially activate matrix metalloproteinases (MMPs) [64, 165, 166] thereby promoting ECM degradation and tumor cell invasion [167]. However, we did not find evidence for upregulation of MMP-9 activity or expression by TGF-β in the MDA-MB-231 cells under the conditions studied.

TGF-β is released from cells mostly in a latent, inactive form via a constitutive secretion pathway [168]. Despite the predominance of latency of TGF-β in conditioned media in general, MDA-MB-231 cells express detectable active TGF-β [63]. It is interesting that uPA can proteolytically activate latent TGF-β [7]. Therefore, TGF-β regulation of uPA production may be a positive feedback loop for activation of latent TGF-β (Fig. 17) and this relationship can be a cycle in cancer progression since TGF-β is over-expressed in both malignant breast tumors and surrounding stroma [169, 170] and uPA expression is increased in human breast carcinomas and bone metastases [171]. It will be of interest to determine whether the basal level of uPA secretion contributes to the availability of active TGF-β and autocrine TGF-β signaling.

The plasminogen activator inhibitor (PAI-1) is a TGF-β target gene and a strong inhibitor of uPA. The present study demonstrates that TGF-β increases levels of both uPA and PAI-1 in the MDA-MB-231 condition media. Interestingly, despite the fact that PAI-1 inhibits uPA activity, concomitant elevation of u-PA and PAI-1 has been observed in breast cancer and is associated with poor outcome [172], suggesting that tumor progression may occur in the

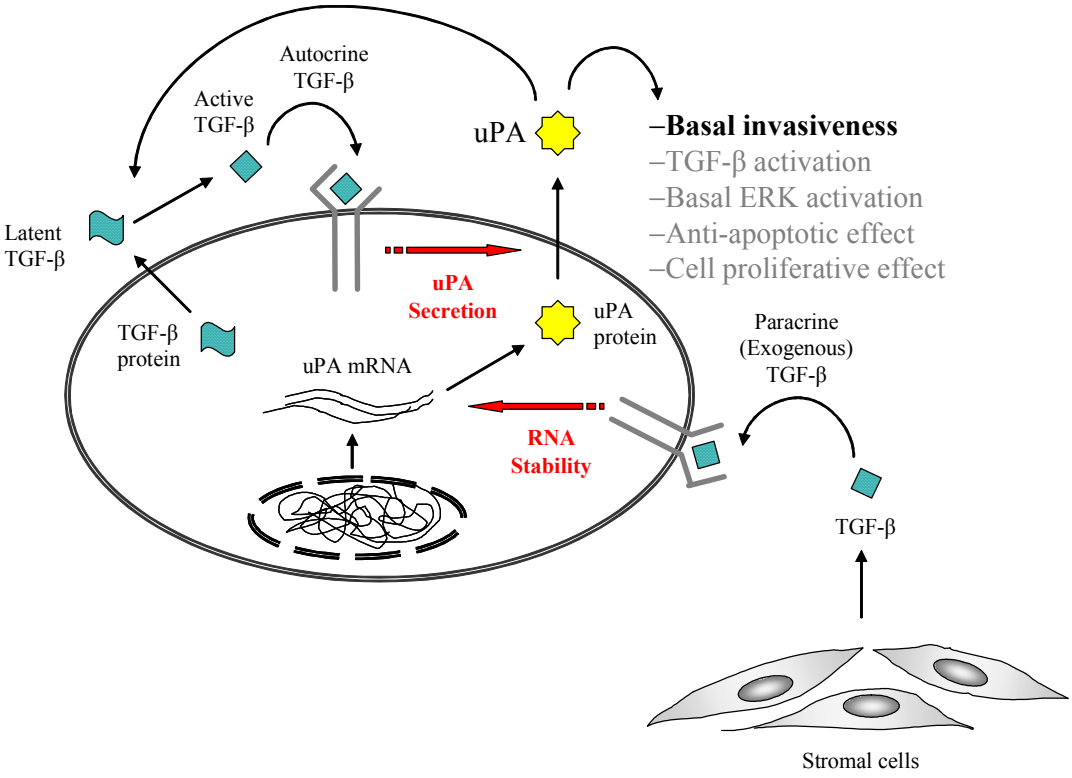


Figure 17. Schematic representative showing: 1) autocrine and paracrine TGF-β modulation of uPA via protein secretion and RNA stabilization, respectively, leading to a more invasive phenotype, 2) a potential positive feedback loop of activation of TGF-β by uPA. Autocrine TGF-β facilitates uPA secretion, which may in turn activate latent TGF-β produced by either tumor or stromal cells, and 3) induction of uPA by autocrine and paracrine TGF-β may cause subsequent ERK activation, loss of apoptotic responses, and increase proliferation that have impact on malignant cellular transformation.

presence of high levels of PAI-1. TGF-β has been shown to stimulate attachment and invasion through up-regulation of PAI-1 [43] and these effects may contribute to cell invasion. Our findings suggest that while PAI-1 is induced by TGF-β, this induction is not sufficient to prevent the invasion promoting effect of uPA.

TGF-β receptor activation results in phosphorylation of Smads2/3 which then form a complex with Smad4, translocate to the nucleus and regulate transcription of target genes [1]. While endogenous levels of Smad2 phosphorylation were below the limit of detection of immunoblot analysis, we found that silencing of Smad4 expression by siRNA was sufficient to inhibit release of uPA into the conditioned medium. Taken together, our results suggest that the basal regulation of uPA secretion in MDA-MB-231 cells is dependent on autocrine TGF-β receptor activation and Smad signal transduction.

In summary, autocrine TGF-β regulates both cell invasiveness and uPA secretion. Inhibition of uPA activity decreases tumor cell invasion by the same extent as inhibiting of autocrine TGF-β signaling, suggesting that autocrine TGF-β regulation of uPA levels associates with the invasiveness. The Smad pathway is necessary to mediate regulation of uPA secretion by autocrine TGF-β. Interestingly, while autocrine TGF-β regulate uPA production through protein secretion, exogenous TGF-β further increases uPA expression through RNA stabilization. Moreover, our study suggests that the efficacy of an inhibitor of TGF-β receptor activation can suppress tumor cell invasiveness and proves the uses for further study of small inhibitors as experimental caner therapeutic agents.

CHAPTER IV

SMAD4 SUPPRESSES CLAUDIN-1 EXPRESSION THROUGH A TGF-β-INDEPENDENT PATHWAY IN COLORECTAL CANCER CELLS

Abstract

Smad4 is a central intracellular signal transduction component of the TGF-β family of cytokines and is considered an important tumor suppressor with the capacity to mediate TGF-β-induced growth inhibition and apoptosis. Clinically, loss of Smad4 protein expression is correlated with poor prognosis and is frequently observed in invasive and metastatic carcinomas of colorectal cancer (CRC). The integral tight junction protein, claudin-1, has been recently observed to be increased in CRC carcinomas and metastases. Moreover, claudin-1 levels are positively correlated with the metastatic potential of CRC cell lines. In the present study, we report an inverse correlation between Smad4 and claudin-1 expression in several CRC carcinomas and cell lines. Smad4 expression in Smad4 deficient, claudin-1-positive HT29 and SW480 cells, resulted in inhibition of claudin-1 protein expression. Expression of other claudins such as claudin-4 and -7 was not altered by expression of Smad4, suggesting the specific regulation of claudin-1 by Smad4. Smad4 expression in SW480 cells inhibited claudin-1 mRNA expression to undetectable levels as determined by RT-PCR and Northern blotting. Moreover, Smad4 expression decreased claudin-1 promoter activity in reporter assays, suggesting transcriptional repression as a mechanism of claudin-1 inhibition by Smad4 expression. The selective TGF-β receptor kinase inhibitor, LY364947, did not prevent the Smad4 suppression of claudin-1 protein expression in either SW480 or HT29 cells suggesting that the Smad4 effect on claudin-1 was independent of TGF-β signaling. Thus, these findings suggest a novel mechanism

underlying Smad4 tumor suppressive function through regulation of a potential metastatic modulator, claudin-1, in a TGF-β independent manner in CRC cells.

Introduction

Smad4 is the central intracellular effector in mediating Smad-dependent signals from the TGF-β family of proteins that are composed of TGF-βs, activins, and bone morphogenetic proteins (BMPs) [173]. This family of proteins regulates a broad range of cellular responses including proliferation, differentiation, migration, and apoptosis during embryonic development and the maintenance of tissue homeostasis in the adult [15]. TGF-β family of proteins initiates signaling by binding and activating membrane serine-threonine kinase receptors. Subsequently, the receptors activate two groups of receptor-associated Smad effectors (Smad2 and 3 for TGF-βs and activins and Smad1, 5, and 8 for BMPs) by phosphorylation. Activated Smads form oligomers with the common Smad effector, Smad4, and then translocate from cytoplasm to the nucleus to regulate gene expression independently or in association with other DNA-binding partners [5]. Smad4 thus has a central role in transducing Smad-dependent signaling from all members of the TGF-β family.

Pathological TGF-β signaling has been implicated in various human diseases such as auto-immune diseases, vascular disorders, and cancer (reviewed in [22]). In tumor-related biology, TGF-β has been focuses of studies as it is a prototype of growth inhibitor and apoptosis inducer in normal epithelial lineages and therefore is considered a cytokine with tumor suppressive activity. Acquisition of resistance to the anti-proliferative effect of TGF-β allows tumor cells to escape from normal growth constraints, which may occur via mechanisms

involving loss or functional inactivation of the components (e.g. loss of the expression of Smad4 or the TGF-β type II receptor) of the TGF-β signal pathway.

Smad4, also termed DPC4 (deleted in pancreatic carcinoma, locus 4), was originally isolated from human chromosome 18q21.1 and is functionally inactivated in approximately half of pancreatic cancers [82], one third of metastatic colorectal carcinomas [84], and smaller subsets of other types of cancer. Concomitant loss of Smad4 expression and acquisition of invasive and metastatic phenotype [86] strongly implicate tumor suppressive functions of Smad4 in tumors. Although Smad4 was initially regarded as a tumor suppressor through mediating TGF-β-induced antiproliferative responses [87, 88], recent evidence has suggested additional tumor suppressive functions of Smad4. For instance, Smad4 expression in Smad4-deficient SW480 colon carcinoma cells resulted in loss of tumorigenicity of these cells in nude mice [87], re-establishment of a more epithelioid phenotype, and increased expression of E-cadherin and P-cadherin [85]. Interestingly, Smad4 restoration did not alter growth inhibitory response to TGF-β, which was attributed to low levels of type II TGF-β receptor resulting in deficient TGF-β signaling [85]. These data suggest that Smad4 may elicit distinct tumor suppressive responses independently of TGF-β signaling.

Disruption of the cell-cell junctions with concomitant changes in the expression of junctional proteins is a critical event in invasive and metastatic progression in cancer. While adherens junction proteins have been studied extensively in cancer, the roles of tight junction (TJ) proteins are less well characterized. Claudins are recently identified members of the tetraspanin family of proteins that are integral to the structure and function of TJs. Altered expression of claudins has been detected in several types of cancers; however, their roles in cancer remain unclear and controversial. We have recently reported elevated claudin-1 expression in colon

carcinomas and metastases and observed a positive correlation between claudin-1 protein levels and tumorigenic and metastatic capacity in colorectal cancer (CRC) cell lines [125]. Therefore, claudin-1 appears to be an important regulator of metastatic transformation of tumor cells.

Given that Smad4 is frequently lost while claudin-1 is over-expressed in metastatic colorectal cancer (CRC) [83, 84, 125], we have investigated their relationship in CRC. In this present study, we report an inverse correlation between Smad4 and claudin-1 expression in CRC carcinomas and cell lines. Furthermore, Smad4 expression in two Smad4-deficient but claudin-1-positive CRC cell lines, HT29 and SW480, suppressed claudin-1 protein expression but did not change expression of other claudins such as claudins-4 and -7. Expression of Smad4 appears to suppress claudin-1 transcriptional activity and markedly suppresses claudin-1 mRNA expression. In SW480 cells, the Smad4 effect on claudin-1 was independent of TGF-β signaling as ectopic Smad4 protein expression was not sufficient to restore TGF-β responsiveness as demonstrated in promoter study using the p3TP-Lux, a luciferase reporter construct that is highly responsive to TGF-β [31], in response to TGF-β. Furthermore, treatment with the TGF-β receptor kinase inhibitor, LY364947, failed to prevent the inhibition of claudin-1 by Smad4 expression in SW480 cells. In HT29 cells, although Smad4 expression restored an autocrine and paracrine TGF-β signaling in terms of promoter activation of the p3TP-Lux in HT29 cells, LY364947 did not prevent the Smad4-mediated suppression of claudin-1 expression, further supporting that Smad4 inhibition of claudin-1 expression occurs independently of TGF-β signaling.

Results

Inverse Claudin-1 and Smad4 expression in colorectal carcinomas and cell lines

Since loss of Smad4 protein expression is associated with advanced disease and is frequently detected in metastatic colorectal cancer (CRC) [84] and claudin-1 has been recently defined as a potential metastatic promoter in CRC cells [125], we examine the correlation between Smad4 and claudin-1 expression in CRC. Expression of claudin-1 and Smad4 was examined in six pairs of normal and tumor tissues. In normal tissues, Smad4 expression was invariably detected but claudin-1 expression is not detected by immunoblotting (Fig 18A). Inverse expression of Smad4 and claudin-1 was observed in five out of the six tumor tissues evaluated (Fig. 18A). In a survey of several colorectal cancer and intestinal epithelial cell lines, claudin-1 protein expression was abundantly detected in the Smad4-deficient SW480, SW620, and HT29 lines, but it was not detectable in Smad4-expressing RIE, HCT 116, and HCT 15 lines (Fig. 18B). Absence of claudin-1 protein expression is correlated with undetectable expression of claudin-1 mRNA as determined by semi-quantitative RT-PCR (Fig. 18B). These data suggest an inverse expression of Smad4 and claudin-1 in colorectal carcinomas and cell lines.

Smad4 reconstitution decreases claudin-1 protein and RNA expression

To determine whether Smad4 can regulate claudin-1 expression, we examined claudin-1 protein levels after Smad4 expression in the Smad4-deficient claudin-1-positive HT29 colon carcinoma cells. Smad4 expression in HT29 cells was achieved by Smad4 adenoviral infection and confirmed by immunoblotting for Smad4. Immunoblotting results show Smad4 expression only in Smad4-adenovirus (Ad-Smad4)-infected but not in parental or β-galactosidase-adenovirus (Ad-β-Gal)-infected HT29 cells (Fig. 19A). Smad4 expression resulted in decreased

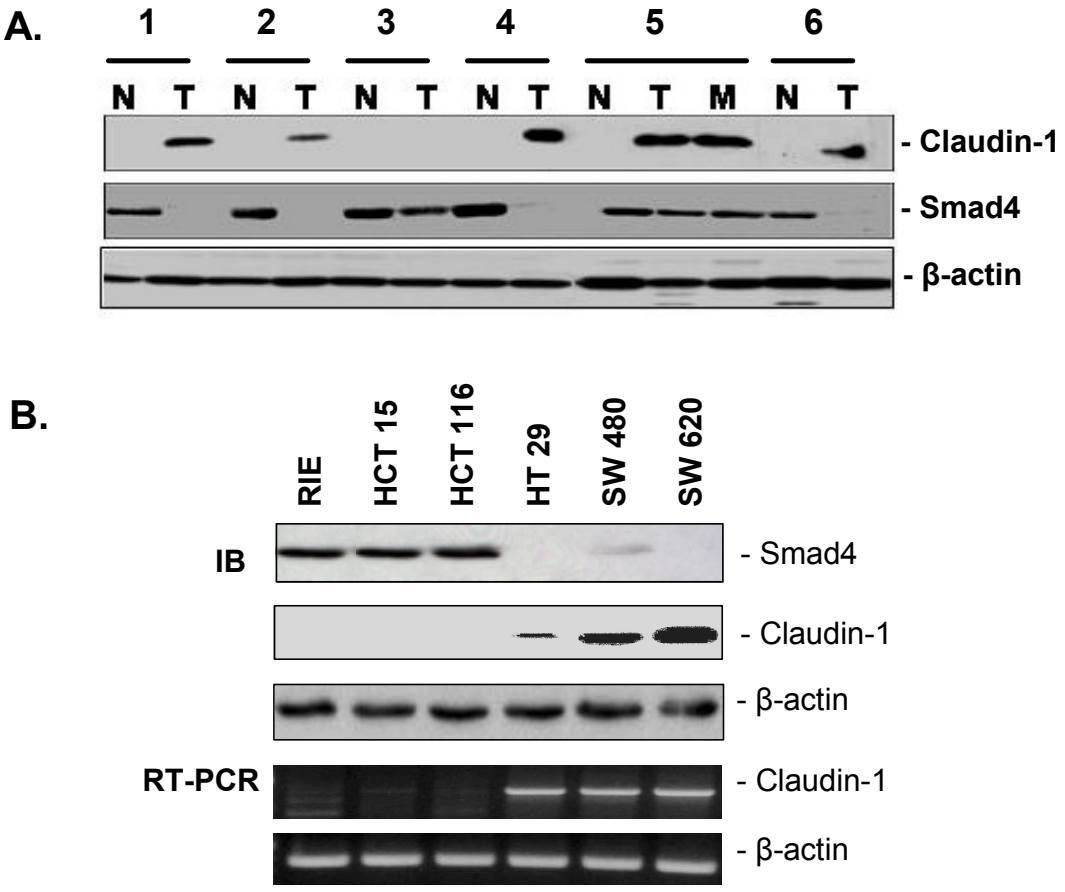


Figure 18. Inverse Smad4 and claudin-1 protein expression in selected colorectal carcinomas and cell lines. (A) Tissue samples were obtained from six patients with colon cancer and designated as "N" for normal mucosa, "T" for primary tumor or "M" for liver metastases. Protein lysates were prepared from homogenized tissues and clarified by centrifugation. Equal amounts of lysates were used in immunoblotting assays for claudin-1, Smad4, and β-actin. (B) Lysates and total RNA from the indicated cell lines were prepared as described in "Materials and Methods" and subjected to immunoblotting (IB) and semi-quantitative RT-PCR (RT-PCR) for claudin-1, Smad4, and β-actin.

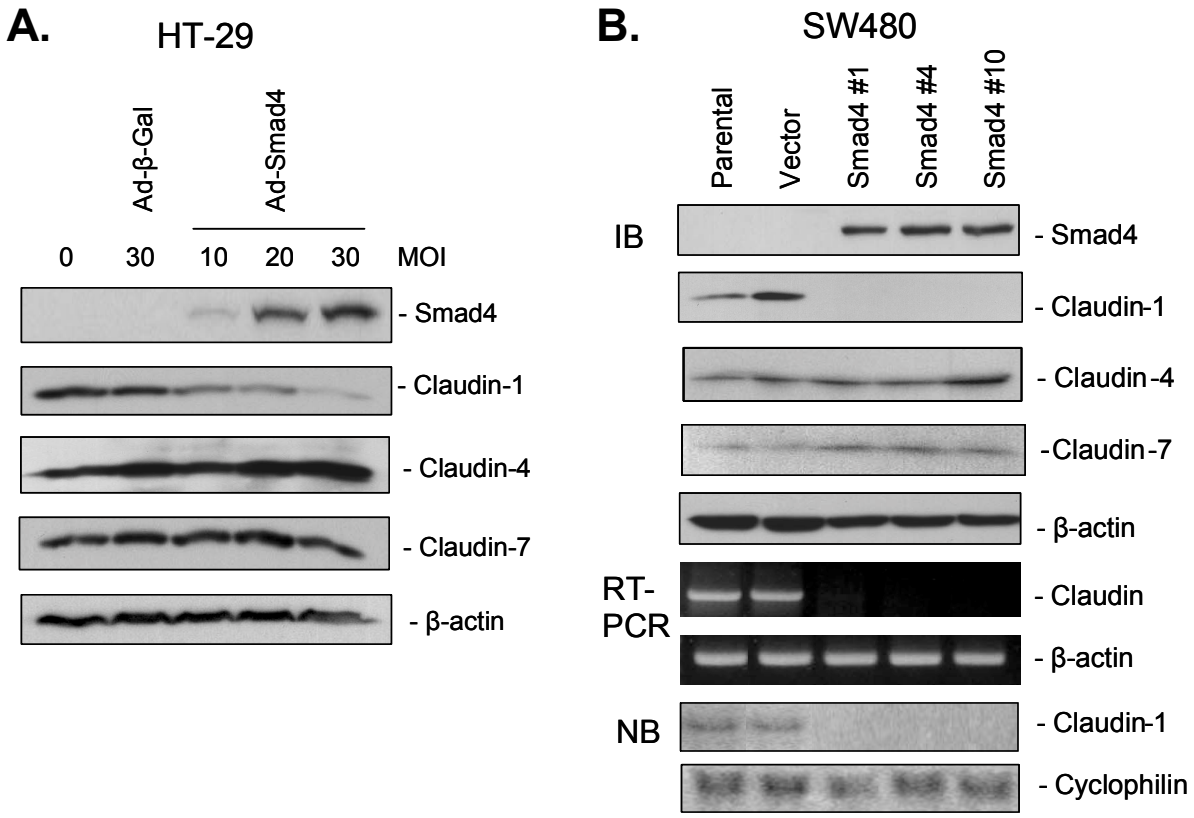


Figure 19. Smad4 expression in Smad4-deficient colon cancer cell lines inhibited claudin-1 protein expression. (A) HT29 cells were infected with β-galactosidase (Ad-β-Gal) or Smad4 (Ad-Smad4) adenoviruses at the indicated MOIs. Seventy two hours later, cells were lysed and the lysates were subjected to immunoblotting assays for the indicated proteins. (B) Protein lysates and total RNA from SW480 parental, vector, and three Smad4-expressing clones were obtained and subjected to immunoblotting (IB) for the indicated proteins, semi-quantitative RT-PCR (RT-PCR) for claudin-1 and β-actin expression, and Northern blotting (NB) for claudin-1 and cyclophilin expression.

claudin-1 expression in a dose-dependent manner, indicating a causal role for Smad4 in the inverse relationship between claudin-1 and Smad4.

To further substantiate these findings and dissect the molecular mechanisms underlying Smad4-mediated inhibition of claudin-1, Smad4-expressing stable clones were derived from the SW480 cells (another Smad4-deficient but claudin-1-positive CRC line). Three clones with stable Smad4 expression were selected and the claudin-1 levels in these clones were determined by immunoblotting. Consistent with the observations in HT29 cells, Smad4 expression resulted in inhibition of claudin-1 expression. This effect of Smad4 appears to be specific for claudin-1 as levels of claudin-4 and -7 proteins did not change after Smad4 expression in both HT29 and SW480 cells (Fig. 17A and B). Consistent with previous findings, Smad4 expression in SW480 cells resulted in a mesenchymal-to-epithelial morphological change, increased E-cadherin, and down-regulation of β-catenin as determined by immunofluorescence and immunoblot studies (Fig. 20A and B). These data show that Smad4 expression profoundly downregulated the expression of claudin-1 in colorectal cancer cells.

Smad4 expression suppresses claudin-1 promoter activity

The absence of detectable steady-state claudin-1 mRNA associated with loss of claudin protein expression (Fig. 19B) suggests suppressed transcription of *claudin-1* by Smad4. To study transcriptional regulation of claudin-1, a reporter assay-based promoter study was performed using a *claudin-1* promoter-reporter construct containing the -1160 to +160 region of the human *claudin-1* promoter or pGL3/-1.2Cld-1 (Fig. 21A). In SW480 parental, vector alone, and three Smad4-expressing clones, luciferase activity of pGL3/-1.2Cld-1 was determined and normalized to that of pGL3-Basic, the backbone of pGL3/-1.2Cld-1. A 34-48% reduction in

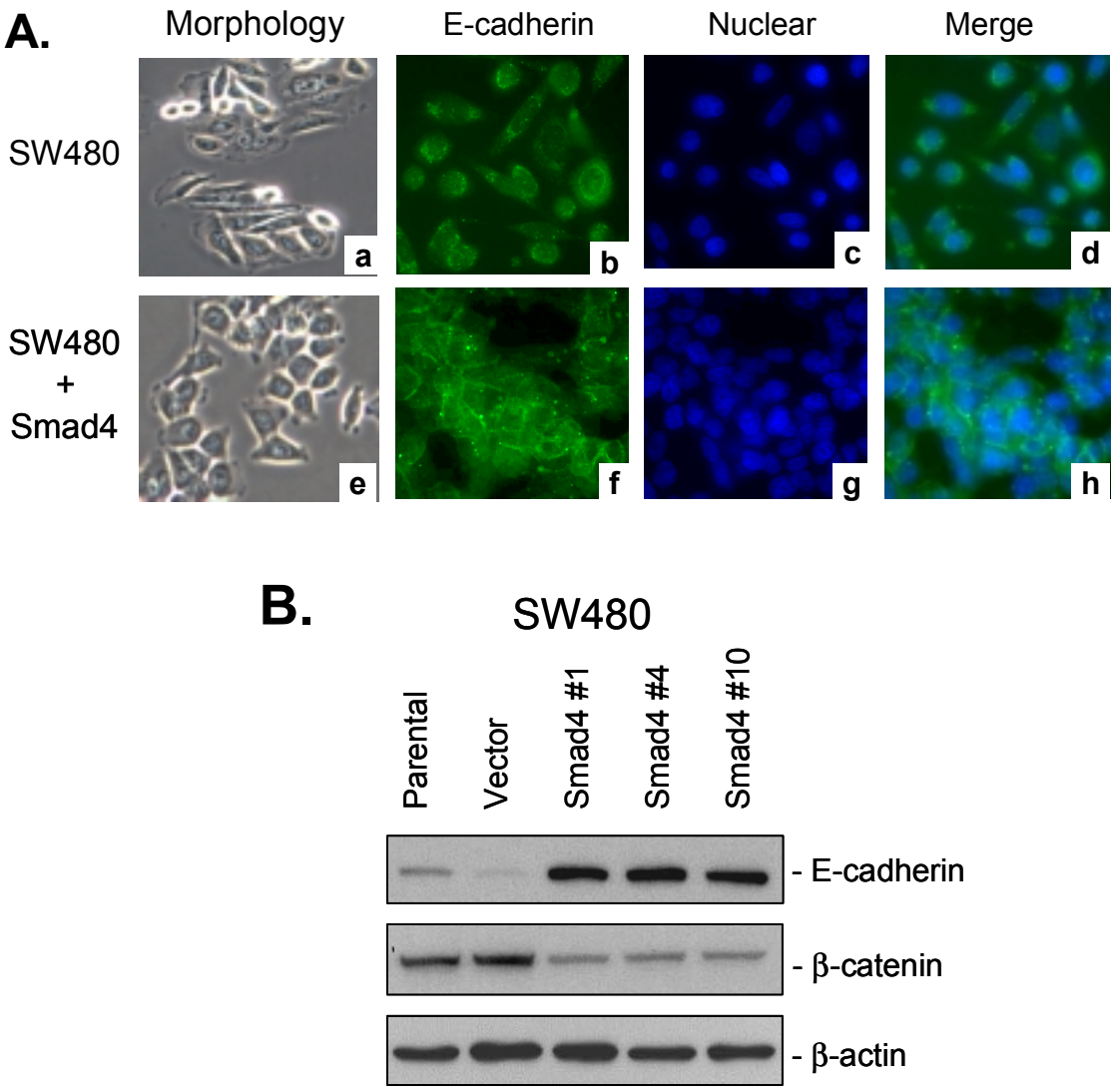


Figure 20. Smad4 re-expression resulted in a mesenchymal-to-epithelial morphological change, increased E-cadherin, and decreased β-catenin protein expression. (A) Parental (a – d) and Smad4-expessing (e – h) SW480 cells grown on multi-chamber slides were photographed under a phase-contrast microscope and then subjected to E-cadherin and DAPI (nuclear) immunofluorescent staining. (B) Smad4 re-expression enhanced E-cadherin but decreased β-catenin protein expression. Whole cell lysates were prepared from SW480 parental, vector, and three Smad4-expessing clones and subjected to immunoblotting for E-cadherin, β-catenin, and β-actin.

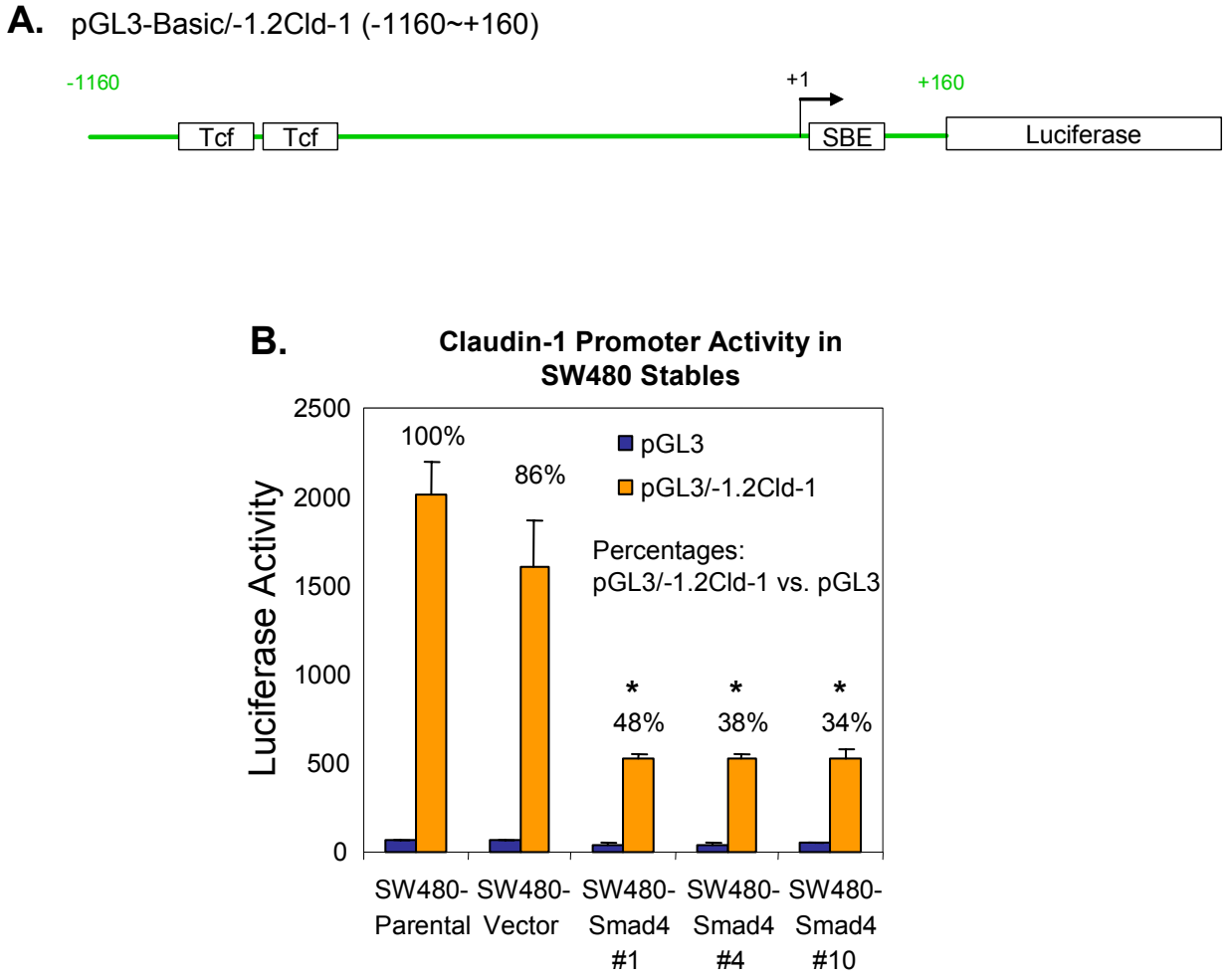


Figure 21. *Claudin-1* promoter activity in parental, vector, and Smad4-expressing SW480 clones. (A) Schematic of the claudin-1 promoter-reporter that contains the -1160 to +160 region of the human claudin-1 promoter (pGL3/-1.2Cld-1). (B) SW480 parental, vector, and three Smad4-expressing clones were transiently transfected with 0.2 μg of pGL3/-1.2Cld-1 or pGL3-Basic (backbone of pGL3/-1.2Cld-1) along with 0.04 μg of a reference reporter (phRL-TK) in triplicate. Forty eight hours after transfection, luciferase activity was determined and plotted as mean ± SD from three independent experiments. Normalized luciferase activity (pGL3/-1.2Cld-1 versus pGL3-Basic) in each individual clone is presented as a percentage (100% in SW480 parental cells). SBE: Smad binding element. *, $P < 0.05$ is derived from one-way ANOVA with a Bonferroni correction.

claudin-1 promoter activity was observed in Smad4-expressing clones when compared with that of SW480 parental cells (Fig. 21B). In contrast, *claudin-1* promoter activity was not significantly reduced in the control vector-stably transfected cells. This inhibitory effect of Smad4 on *claudin-1* promoter activity was also observed in transient co-transfection experiments in SW480 and SW620 cells, in which cotransfection of Smad4 decreased *claudin-1* promoter activity (Fig 22A and B). Thus, Smad4 appeared to suppress claudin-1 expression through transcriptional control and the necessary *cis*-elements are within the -1160 to +160 proximal area of the *claudin-1* promoter.

TGF-β signaling is not recovered by Smad4 expression and is not required for Smad4 suppression of claudin-1 expression in SW480 cells

SW480 cells secrete TGF-β [174]. To determine whether ectopic Smad4 expression restores TGF-β responsiveness establishing autocrine and paracrine TGF-β signaling and to determine whether TGF-β signaling is necessary for the effect of Smad4 expression on claudin-1 expression in the SW480 cells, we sought evidence for TGF-β signaling. This was done by examining nuclear accumulation of Smad4 and Smad2. Intact TGF-β signaling results in translocation of the Smad2/3 and Smad4 complex to the nucleus in response to receptor activation. Immunofluorescence confirms the expression of Smad4 in the selected SW480 stable clones but not in the parental cells (Fig. 23B). Immunofluorescence showed no significant nuclear-localization of Smad2 in either parental or Smad4-expressing SW480 clones (Fig. 23A). Interestingly, the Smad4 staining was predominantly in the nucleus despite the absence of Smad2 nuclear localization (Fig. 23B).

We next examined whether Smad4 restores TGF-β-mediated promoter activation of p3TP-Lux, a luciferase reporter construct that is highly responsive to TGF-β [31]. Our results

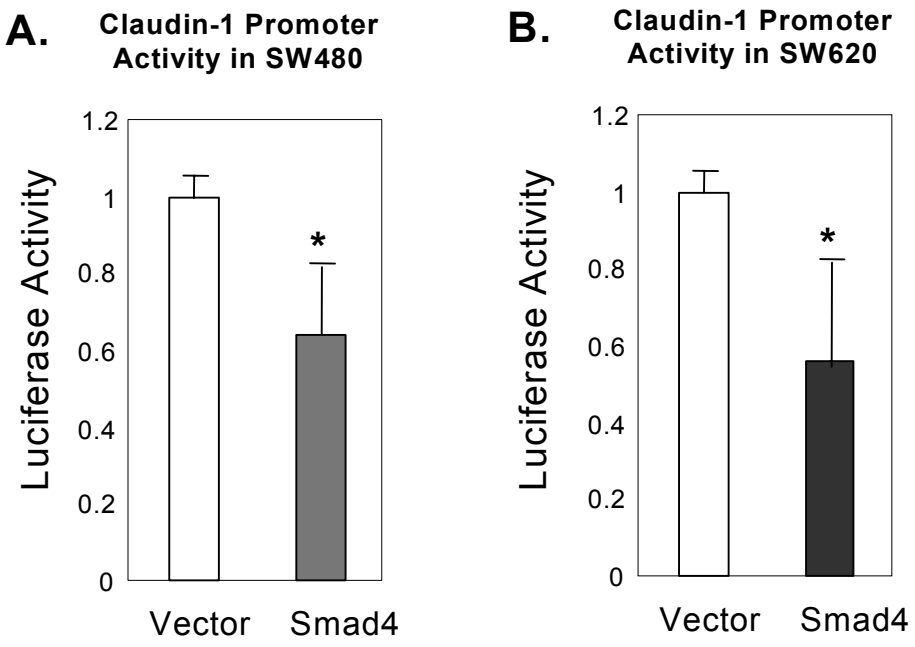


Figure 22. Smad4 expression decreased *claudin-1* promoter activity. SW480 (A) and SW620 (B) cells were co-transfected with 0.05 μg of a claudin-1 promoter-reporter (pGL3/-1.2Cld-1), 0.04 μg of a reference reporter (phRL-TK), and 0.15 μg of a control or a Smad4 expression vector. Reporter assay was performed 72 hours after transfection. *, $P < 0.05$ is derived from t test; bars, standard deviation.

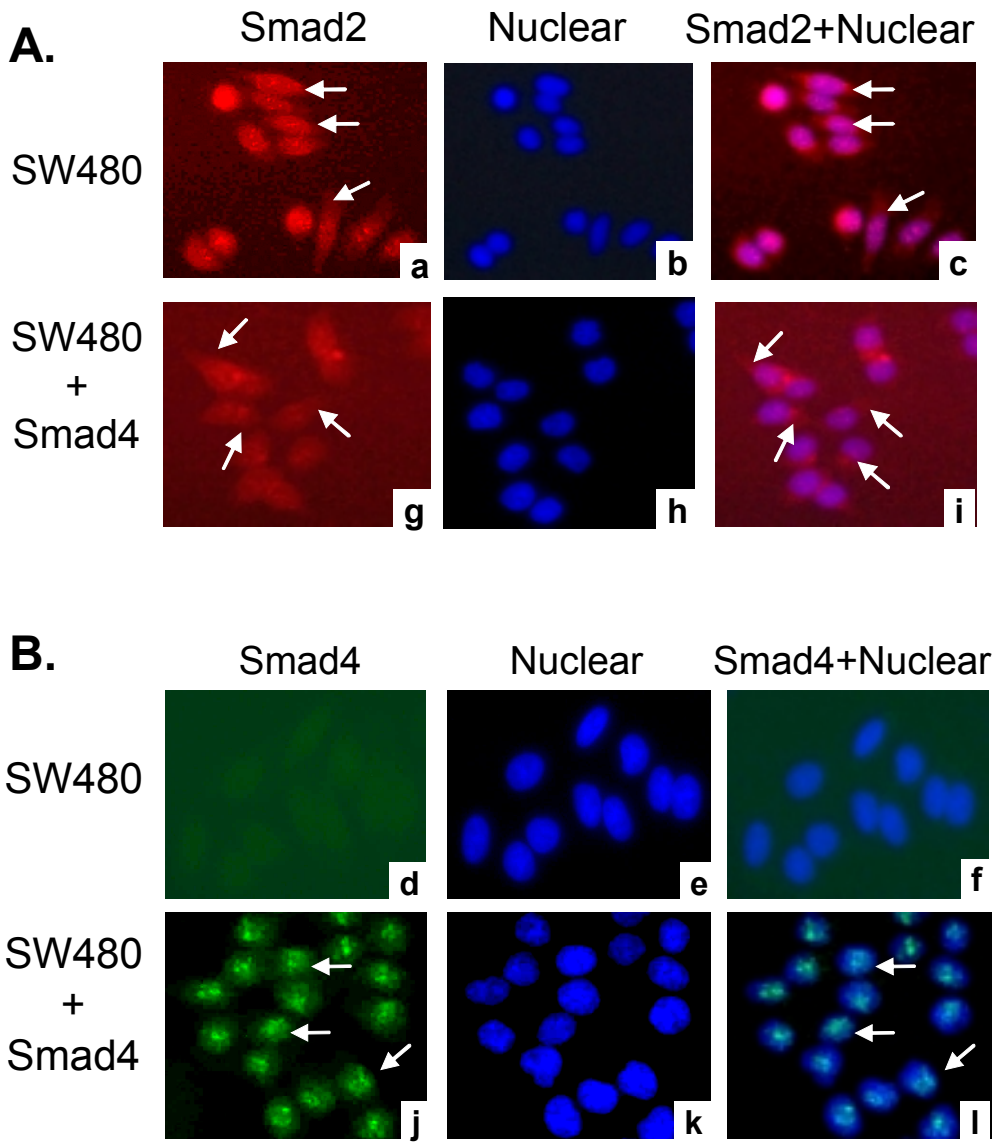


Figure 23. Immunofluorescent staining for Smad2 (A) and Smad4 (B) in parental (a-c and d-f) and Smad4-expessing (g-i and j-l) SW480 cells. Parental and Smad4-expessing SW480 cells grown on multi-chamber slides were subjected to immunofluorescent staining for Smad4, Smad2, and DAPI (nuclear) and photographed under an up-right fluorescent microscope.

showed no induction of p3TP-Lux activity by TGF-β treatment in SW480 parental, vector, or Smad4 expressing clones (Fig. 24). SW480 cells are known to express very low levels of the TGF-β type II receptor (TβRII) [85], which may explain why expression of Smad4 did not restore TGF-β responsiveness. This hypothesis was further supported by transient co-transfection experiments showing that TGF-β responsiveness was restored when TβRII was co-expressed in Smad4-expressing SW480 (Fig. 24). Thus, expression of Smad4 alone neither restored responsiveness to exogenous TGF-β nor established an autocrine TGF-β loop in SW480 cells. Therefore, TGF-β signaling may not be required for the Smad4 effect on claudin-1 expression in these cells. This conclusion was further supported by experiments using a TGF-β receptor kinase inhibitor, LY364947. LY364947 is a potent ATP competitive inhibitor of the TGF-β type I and II receptors (TβRI and TβRII) and inhibits the kinase activity of the TGF-β receptors and TGF-β signaling [29]. Immunoblotting shows that LY364947 treatment did not prevent the inhibition of claudin-1 expression by Smad4 expression in the SW480 cells (Fig. 25).

TGF-β response is enhanced by ectopic Smad4 expression and is not required for Smad4 suppression of claudin-1 in HT29 cells

HT29 is another cell line in which we observed the Smad4 inhibitory effect on claudin-1 expression. In contrast to SW480 cells, HT29 cells express sufficient levels of TGF-β type II receptor (TβRII) to transduce TGF-β-mediated Smad2 phosphorylation [175]. Nevertheless, TGF-β signaling to the nucleus is defective due to the deficiency of Smad4 expression (Fig. 18B, 19A, and 26A). In transient co-transfection experiments using, we found that TGF-β activated p3TP-Lux promoter in Smad4 co-transfected cells (Fig. 26A). Of note, Smad4 co-transfection enhanced basal p3TP-Lux promoter activity, suggesting the establishment of

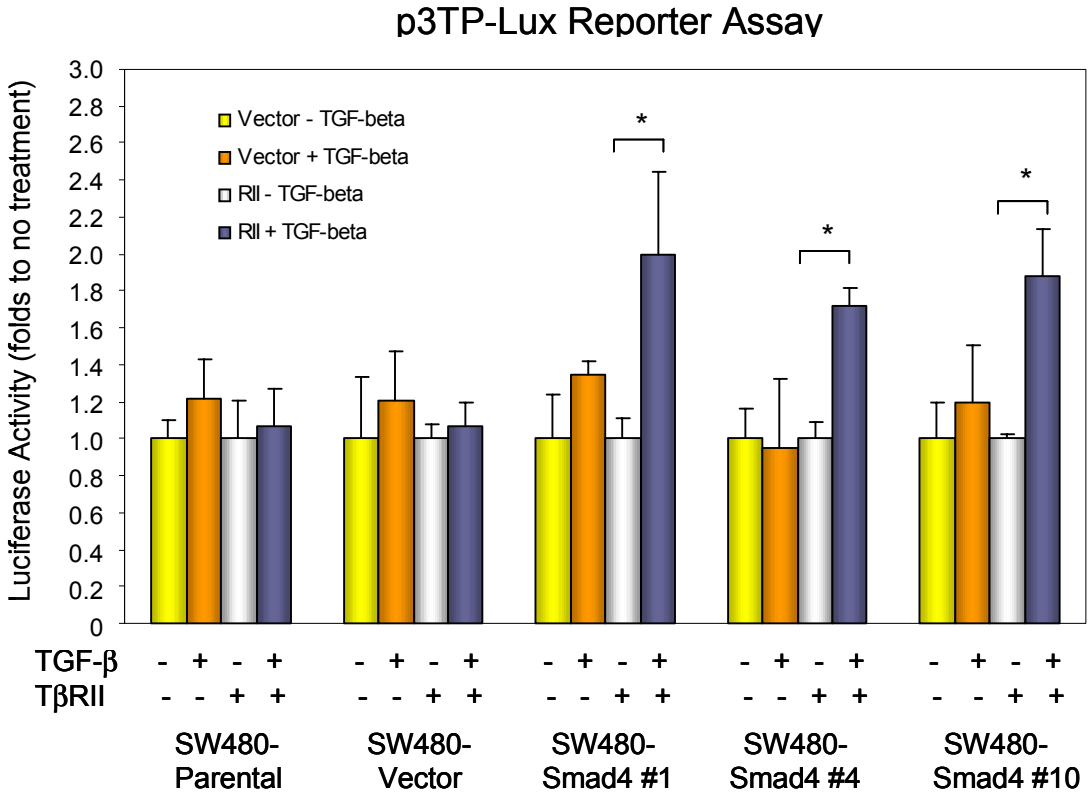


Figure 24. The TGF-β signaling pathway was not restored by Smad4 expression but was restored by co-expression of Smad4 and wild-type TGF-β type II receptor (RII) in SW480 cells. SW480 parental, vector, and Smad4 clones were transiently co-transfected with 0.05 μg p3TP-Lux, 0.04 phRL-TK, and 0.15 μg of a control vector or a RII expression vector. Sixteen hours after transfection, cells were treated with 3 ng/ml TGF- β and allowed to grow for 48 hours before luciferase assays. Luciferase activity was plotted as mean ± SD from three independent experiments and represented as fold change (TGF-β treatment versus vehicle treatment) in each individual line. *, $P < 0.05$ is derived from t test (treatment vs. no treatment); bars.

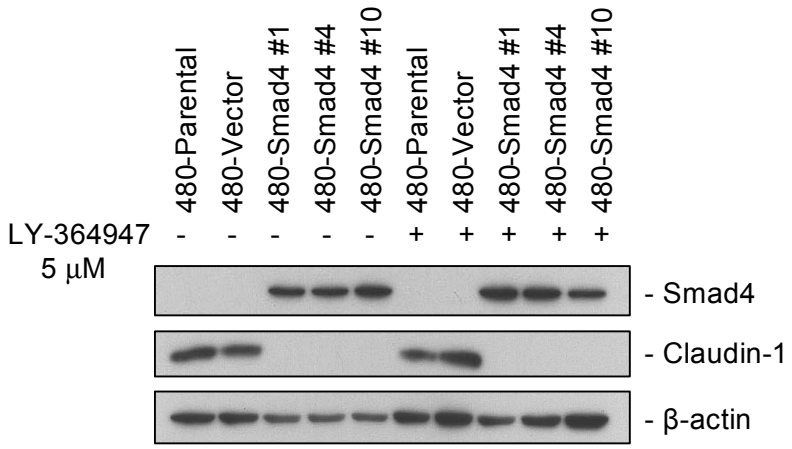


Figure 25. The TGF-β receptor kinase inhibitor, LY364947, did not affect Smad4-mediated suppression of claudin-1 expression in SW480 cells. SW480 parental, vector, and three Smad4-expressing clones were grown in the presence of vehicle or 5 μM of LY364947 for 48 hours and then lysed. Equal amounts of cell lysates were used for immunoblotting for Smad4, claudin-1, and β-actin.

autocrine TGF-β signaling by Smad4 expression. Although LY364947 inhibited basal and TGF-β-induced p3TP-Lux promoter activation in Smad4 co-transfected cells (Fig. 26A), it did not prevent Smad4-mediated suppression of claudin-1 expression in HT-29 cells (Fig. 26B), confirming that the effect of Smad4 on claudin-1 expression is independent of TGF-β signaling in CRC cells.

Autocrine BMP signaling is increased by Smad4 restoration

As described above, Smad4 alone did not restore TGF-β signaling in SW480 cells and the Smad4 effect on claudin-1 did not require TGF-β signaling in both SW480 and HT29 cells. Interestingly, Smad4 was detected primarily in the nucleus of Smad4-expressing SW480 clones (Fig. 23B). It is possible that another Smad4-dependent TGF-β related signaling pathway, such as BMP signaling, may be uncovered by Smad4 expression. Intracellular localization of BMP-related Smads (Smad1, 5, and 8) and phospho-Smad1, 5, and 8 (activated forms) were examined. Immunofluorescence showed both cytoplasmic and nuclear BMP Smads in parental and Smad4 clones, whereas phospho-Smad1, 5, and 8 are primarily localized in the nuclei of these cells (Fig. 27A). As SW480 parental cells express low but detectable Smad4 protein (Fig. 18B), we can not rule out the possibility of existence of an active BMP signaling in parental cells. Interestingly, we detected a substantial increase in BMP-induced promoter activation in a transient co-transfection experiment using a BMP-responsive reporter (BRE2-Luc) in SW480 parental cells (Fig. 27B). Consistently, BMP signaling was also elevated in SW480 Smad4 stable cells as compared with vector controls (Fig. 27C). The increase in BRE2-Luc activity in the absence of exogenous BMP suggests that autocrine BMP signaling is activated by expression of Smad4 in the SW480 cells. These data indicate that Smad4 reconstitution enhanced autocrine BMP

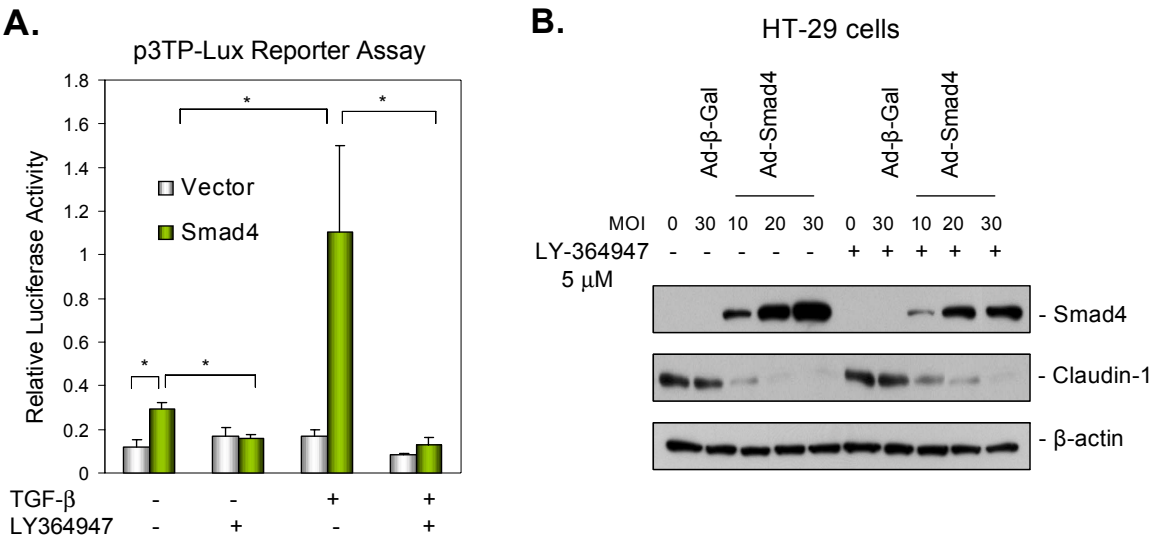


Figure 26. The TGF-β signaling pathway was restored by Smad4 expression but was not required for Smad4-mediated suppression of claudin-1 expression in HT29 cells. (A) The TGF-β receptor kinase inhibitor LY364947 inhibited Smad4-restored autocrine and paracrine TGF-β signaling pathways. Cells were transiently co-transfected with 0.1 μg p3TP-Lux, 0.01 μg phRL-TK, and 0.4 μg of a control vector or a Smad4 expression vector. Sixteen hours later, cells were left untreated or treated with 5 μM LY364947, 3 ng/ml TGF-β, or in combination for 48 hours. Cells were then lysed and subjected to reporter assays. (B) The TGF-β receptor kinase inhibitor, LY364947, did not abrogate Smad4 suppression of claudin-1 in HT29 cells. Cells were infected with β-galactosidase (Ad-β-Gal) or Smad4 (Ad-Smad4) adenoviruses at the indicated MOIs for 3 hours and then cultured in the presence or absence of 5 μM LY364947 for 72 hours. Lysates were prepared and subjected to immunoblotting for Smad4, claudin-1 and β-actin. *, $P < 0.05$ is derived from one-way ANOVA with a Bonferroni correction; bars, standard deviation.

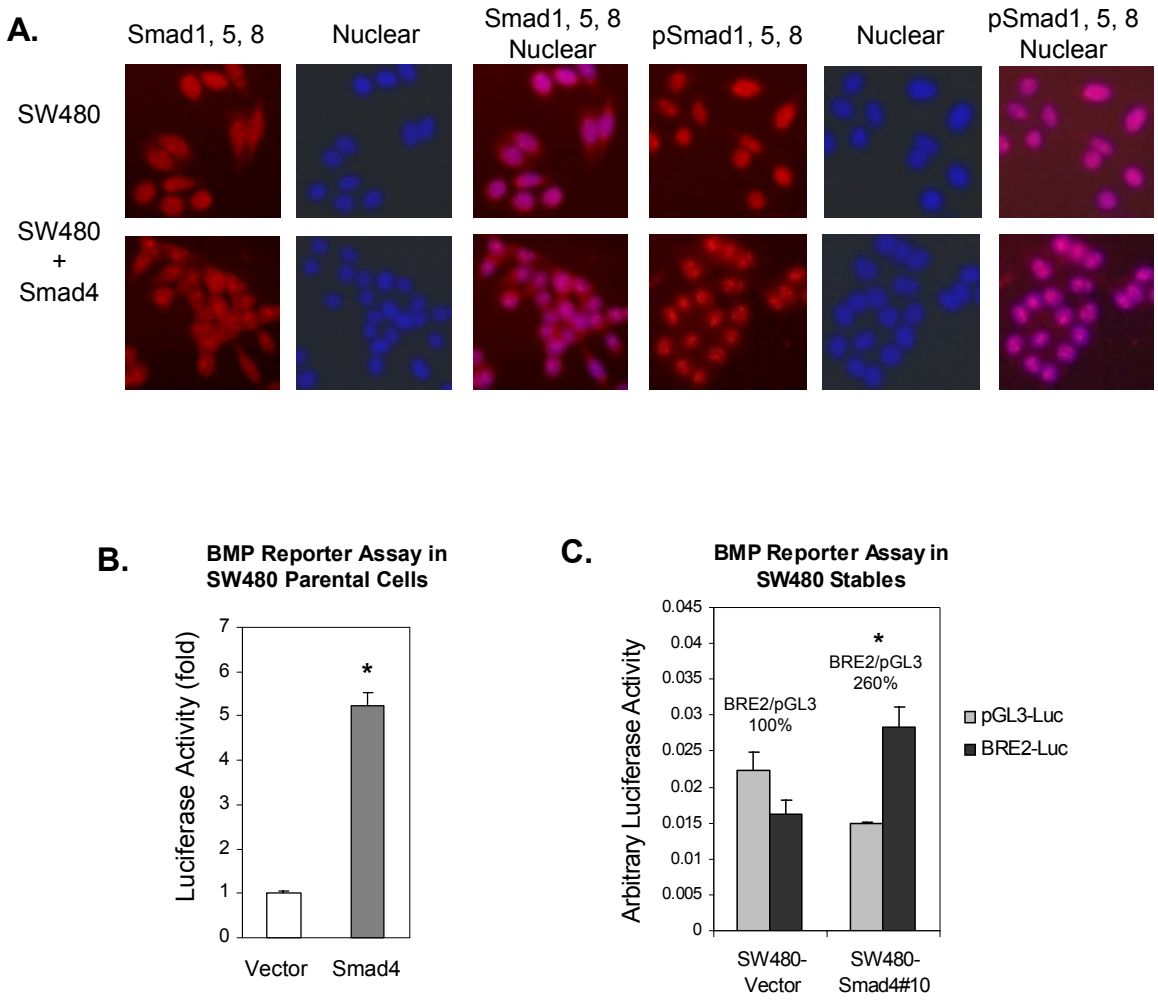


Figure 27. Smad4 re-expression enhanced autocrine BMP signaling in SW480 cells. (A) Intracellular localization of Smad1, 5, and 8 (BMP-associated Smads) and phosphor-Smad1, 5, and 8 in parental and Smad4-expressing SW480 cells by immunofluorescent staining. (B) Smad4 expression enhanced the promoter activity of a BMP-responsive reporter (BRE2-Luc). SW480 cells were co-transfected with BRE2-Luc and either a pCMV-Smad4 or a control vector along with a reference reporter construct. Luciferase activity was measured 48 hours after transfection. (C) Elevated BMP signaling in stable Smad4-expressing SW480 cells. Vector-containing or Smad4-expressing SW480 cells were transiently co-transfected with either BRE2-Luc or pGL3-Basic (backbone of BRE2-Luc) and phRL-TK. Forty eight hours later, luciferase activity was measured and plotted as mean ± SD of three independent experiments. Normalized luciferase activity (BRE2-Luc versus pGL3-Basic) is presented as a percentage (100% in vector control cells). *, $P < 0.05$, is derived from t test; bars, standard deviation.

signaling. Whether BMP signaling is involved the Smad4 effect on claudin-1, E-cadherin, and reversal of EMT is currently under investigation.

Discussion

Smad4 is a key signal transduction mediator for the TGF-β family of cytokines. Its cellular functions are commonly investigated as a central player of this signaling pathway and a tumor suppressor. Loss of Smad4 function has been linked to advanced disease and worse prognosis in colorectal cancer [84, 86]. Smad4's tumor suppressor functions have been linked to TGF-β-induced growth inhibition and apoptosis. Interesting, Smad4 expression in Smad4-deficient cells results in increased E-cadherin expression, reversal of EMT, reconstitution of intercellular adhesions, and loss of *in vivo* tumorigenicity of the cells [176, 177]. These findings provide evidence for additional tumor suppressive functions of Smad4.

The tight junction protein claudin-1 is increased in CRC carcinomas and metastases and plays a role in the regulation of metastatic progression in CRC cells [125]. In the current study, we report that Smad4 is a negative regulator of claudin-1 possibly through transcriptional control in the colorectal carcinoma cells SW480 and HT29. Of note, SW480 cells with low endogenous Smad4 protein expressed lower levels of claudin-1 than SW620 cells, which expressed undetectable Smad4 by immunoblotting. These two lines were derived from the same patient with CRC (SW480 from primary carcinoma and SW620 from a liver metastasis) [178] and showed differential metastatic potential in nude mice, positively correlating with claudin-1 levels in our recent study [125]. Therefore, data obtained from these two lines further support the observation that there is an inverse relationship between Smad4 and claudin-1. The downregulation of claudin-1 by Smad4 may be one of the mechanisms by which Smad4 functions as a tumor suppressor in gastrointestinal epithelium.

In studying the requirement of TGF-β signaling for the Smad4 effect, we observed that Smad4 expression was not sufficient to establish an autocrine TGF-β loop nor did it restore responses to exogenous TGF-β unless TGF-β type II receptor was co-expressed in SW480 cells. In contrast to SW480 cells, Smad4 reconstitution restored the TGF-β response in HT29 cells, suggesting that both TβRII and Smad4 are the limiting factors in SW480 cells while Smad4 limits TGF-β responsiveness in HT29 cells. In these two cell culture models, the profound effect of Smad4 on claudin-1 expression was not affected by blocking TGF-β signaling with the TGF-β receptor kinase inhibitor LY364947.

Interestingly, in the absence of active TGF-β signaling in SW480 cells, Smad4 was predominantly localized to the nucleus, an indication of active Smad signaling. As Smad4 is the common effector in transmitting Smad-dependent signaling from all members of the TGF-β family of cytokines, it is possible that another Smad4-dependent TGF-β-related signaling pathway that does not utilize Smad2 and 3 as effectors, namely bone morphogenetic protein (BMP) signaling, may be uncovered by Smad4 expression. A functional assay of BMP signaling using a reporter construct containing BMP-responsive elements in the promoter area showed significant increases in basal BMP signaling upon Smad4 expression. Interestingly, BMP-7 has been shown to reverse TGF-β-induced EMT in renal tubular epithelial cells and mammary ductal epithelial cells through a Smad-dependent pathway [179]. Therefore, it is possible that BMP signaling is involved in Smad4-mediated reversal of EMT and suppression of claudin-1 in our cell model systems.

E-cadherin expression is particularly important in tumor suppression as it maintains epithelial integrity and restrains cells from movement through tight cell-cell contacts. In addition, its expression results in recruiting and antagonizing the activity of β-catenin, a known proto-

oncogene downstream of Wnt signaling as well as a positive regulator of claudin-1 [124]. Beta-catenin regulates target gene expression by functioning as a co-activator of the transcription factor Tcf/Lef-1 (T cell factor/lymphoid enhancer-binding factor-1) [180]. Therefore, re-appearance of E-cadherin upon Smad4 restoration [85] (Fig. 18A and B) may impair transcriptional activity of β-catenin/Tcf, leading to altered gene expression in SW480 cells. Indeed we have performed a functional assay to determine the transcriptional activity of β-catenin/Tcf/Lef-1 by transient expression experiments using reporters that have either wild-type (TOP-Flash) or mutant β-catenin/Tcf (FOP-Flash) responsive elements in the promoters. In SW480 parental, vector, and Smad4 stable clones, significantly decreased β-catenin/Tcf activity was only detected in the Smad4 clones (data not shown). Given that claudin-1 is a target gene of β-catenin/Tcf and that the β-catenin/Tcf elements are present within the -1160 to +160 region of the *claudin-1* promoter appeared to be sufficient to mediate Smad4-inhibited promoter activity, impaired Wnt signaling may contribute to the transcriptional inhibition of claudin-1.

The *claudin-1* promoter has a canonical Smad binding element (SBE) in the -1160 to +160 area (Fig. 3A) and is negatively regulated by Smad4 in SW480 cells. Smads bind DNA with low affinity and specificity [15, 173] and therefore, they generally need to interact with other transcription factors or co-factors to achieve specific transcriptional regulation. For instance, Smad4 is able to inhibit the promoter activity of selected genes in association with transcriptional co-repressors such as Nkx3.2 [181], TGIF, and the Ski family of proteins [81, 182] that are able to recruit other co-repressors or histone deacetylases. It will be important to determine whether Smad4 regulation of claudin-1 transcription involves similar mechanisms.

Taken together, our findings demonstrate a novel TGF-β independent mechanism for the tumor suppressive functions of Smad4 through the inhibition of claudin-1 expression.

CHAPTER V

SIGNIFICANCE

In this dissertation, my work has extended our understanding of the roles of TGF-β and Smad4 in tumor promotion and suppression. In "Autocrine transforming growth factor-β regulates urokinase plasminogen activator and invasiveness in breast cancer cells", a molecular mechanism underlying the tumor promoting effect of autocrine TGF-β has been identified. My studies demonstrate that tumor cells can be self-sufficient in maintaining a malignant phenotype by producing TGF-β, which then acts in an autocrine manner to promote a regulator of the activity of ECM-degrading enzymes, urokinase plasminogen activator (uPA) thereby maintaining tumor cell invasive growth. Interestingly, autocrine TGF-β regulates uPA through protein secretion, whereas paracrine TGF-β stimulates uPA expression by stabilizing mRNA, suggesting distinct molecular mechanisms of regulation of gene (uPA) expression in response to different magnitudes of stimulation (TGF-β). Furthermore, although Smad4 is predominately considered as a tumor suppressor, it mediates TGF-β-induced uPA production with a tumor promoting effect in breast cancer cells. My work also demonstrates the efficacy of a TGF-β receptor kinase inhibitor in suppressing tumor cell invasiveness providing evidence for the potential of a small-molecule TGF-β signaling inhibitor for therapeutic intervention in cancer.

Claudin-1 is an integral tight junction protein that we recently found to be increased in CRC carcinomas. Elevated expression of claudin-1 is correlated with the metastatic potential of CRC cell lines. In the "Smad4 suppresses Claudin-1 transcription in colorectal cancer in a TGF-β-independent pathway" study, Smad4 expression in Smad4-deficient, claudin-1-positive HT29

and SW480 cells resulted in inhibited expression of claudin-1. Furthermore, the Smad4 effect on claudin-1 expression was independent of TGF-β signaling. Smad4 was initially regarded as a tumor suppressor through mediating TGF-β-induced antiproliferative responses. My study suggests a novel mechanism underlying Smad4 tumor suppressive functions through regulation of a potential metastatic modulator, claudin-1 and this Smad4 effect does not require TGF-β signaling.

In summary, my studies have provided further understanding of 1) the molecular mechanism by which tumor cells maintain malignant phenotype by producing cytokine to promote their own invasive capacity in an autocrine manner, 2) a role for Smad4 in mediating TGF-β pro-tumorigenic effect, and 3) a novel TGF-β signaling-independent mechanism underlying Smad4 tumor suppressive functions.

CHAPTER VI

FUTURE DIRECTIONS

Smad4 restoration in SW480 colon carcinoma cells resulted in loss of tumorigenicity in nude mice, reversal of EMT, re-expression of E-cadherin [85, 87], and downregulation of claudin-1. The effect of Smad4 expression on claudin-1 in these cells is independent of TGF-β signaling. Smad4 restoration elevated endogenous BMP signaling in these cells (Fig. 24). BMP has been implicated in reversal of EMT [179]. It will be of interest to determine whether downregulation of claudin-1 mediates loss of tumorigenicity in nude mice, reversal of EMT, re-expression of E-cadherin after Smad4 restoration in SW480 cells, the mechanisms of how claudin-1 is regulated by Smad4 transcriptionally, and whether BMP signaling is involved in Smad4 regulation of claudin-1.

Determine whether Smad4 regulates EMT and tumorigenicity through suppression of claudin-1 expression in SW480 cells

Epithelial and mesenchymal markers (e.g. E-cadherin and vimentin, respectively) and *in vivo* tumor formation (tumor size and incidence) will be determined with or without forced expression of claudin-1 in Smad4-expressing stable SW480 clones. Specifically, one of the Smad4-expressing SW480 stable clones (SW480:Smad4) with an inducible claudin-1 gene (SW480:Smad4:i-claudin-1) under the control of tetracycline or doxycycline (Tet-Off system) will be generated. Claudin-1 protein expression in response to doxycycline will be determined by immunoblotting. Expression of E-cadherin and vimentin proteins will be examined in SW480:Smad4:i-claudin-1 with (in the absence of doxycycline) or without (in the absence of

doxycycline) claudin-1 expression by immunoblotting. If forced claudin-1 expression prevents reversal of EMT induced by Smad4 expression, tumorigenicity of SW480:Smad4:i-claudin-1 with or without forced claudin-1 expression will be determined. SW480:Smad4:i-claudin-1 cells will be subcutaneously injected into nude mice, which will be treated with or without doxycycline (2 mg/kg) in their drinking water. Tumor growth will be assessed non-invasively at 2-week intervals by contrast enhanced CT imaging. Incidence of tumor formation and tumor size will be assessed at autopsy when the tumor mass approaches 20% of the body mass. Claudin-1 and Smad4 protein expression will be examined in tumors by immunoblotting.

Determine the molecular mechanism for Smad4 suppression of claudin-1 transcription

Rational: The claudin-1 promoter has a canonical Smad binding element (SBE) (Fig. 26) and is negatively regulated by over-expression of Smad4 in SW480 cells. Smad4 is known to inhibit gene expression by suppressing promoter activity in association with transcriptional co-repressors, such as Nkx3.2, TGIF, and Ski, that recruit still other co-repressors or histone deacetylases [81, 181].

Define the region(s) in the claudin-1 promoter required for the suppression of claudin-1 transcription by Smad4

In the result sections, a ~1.3 kb region of the claudin-1 promoter was shown to be sufficient to mediate Smad4 suppression of claudin-1 promoter activity. Promoter studies will be conducted using reporters directed by a series of 5'deletions of the 1.3 kb *claudin-1* promoter (Fig. 26) to identify area(s) necessary for transcriptional regulation by Smad4. Consecutive 5' deletions will be generated by PCR using four forward primers and a single reverse primer and then cloned into pGL3-Basic vector. Promoter activity of these reporters and pGL3-Basic/-

1.3hCla-1 will be determined and compared with SW480:Smad4 cells. We will also perform assays utilizing promoter-reporter constructs with mutation(s) in the identified region to confirm the specificity of the sequence required for the Smad4-dependent regulation of claudin-1 transcription.

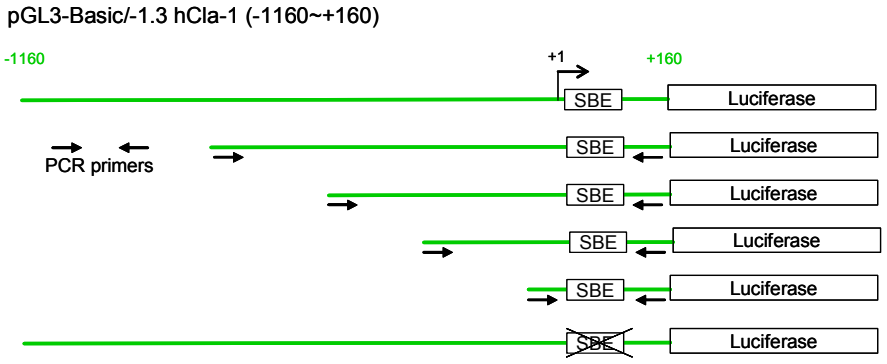


Fig. 28**:** Schematic diagram showing promoter-reporter constructs containing 5' serial deletions or mutations of the *claudin-1* promoter, location of canonical Smad binding elements (SBEs) in the human *claudin-1* promoter, and primers for generation of a series of 5' deletion of the 1.3 kb upstream region of the *claudin-1* promoter.

Determine Smad4 interaction with the *claudin-1* promoter

To test whether Smad4 binds to the SBE in the *claudin-1* promoter *in vitro*, gel shift assays will be conducted using nuclear extracts of SW480 parental, SW480:vector, and SW480:Smad4 clones and radiolabeled SBE oligonucleotides. Specific protein and DNA interaction will be confirmed using a molar excess of either cold or hot mutant SBE oligonucleotide competitors. The presence of Smad4 in the protein/DNA complexes will be determined by super shift assays using Smad4 antibody and IgG (as a negative control).

After assessing *in vitro* Smad4 binding to the SBE, chromatin immunoprecipitation (ChIP) assays will be conducted to determine cellular *in vivo* binding of Smad4 to the *claudin-1*

promoter. Briefly, SW480 variants in media will be incubated with 1% formaldehyde in the media to cross link proteins and DNA *in vivo*. Cells will then be lysed and subjected to sonication to obtain chromatin complexes containing ~500 bp genomic DNA fragments. Pellets of the chromatin complexes will be collected from the lysates by centrifugation, dissolved in a ChIP assay buffer, and then subjected to Smad4 immunoprecipitation using a Smad4 antibody (no addition of antibody will be included in experiments as a negative control) and protein A/G beads. The DNA-protein cross-links in the immune complexes eluted from beads will be separated by a 12 hour-incubation at 65°C and then subjected to protein and RNA digestion. Subsequently, DNA will be purified and subjected to PCR using primers flanking the SBE site of the human *claudin-1* promoter. Chromatin complexes before IP or IP with IgG will be included in experiments as positive and negative controls, respectively.

Identify transcriptional co-repressors associated with Smad4

Smads may negatively regulate promoter activity of certain genes in association with transcriptional co-suppressors [81, 181]. If Smad4 *in vivo* binds to the *claudin-1* promoter, we will examine the association between Smad4 and known Smad-binding co-repressors. Smad4 immunosdprecipitation using the nuclear extracts of SW480 variants followed with immunoblotting for Nkx, TGIF, Ski, and SnoN will be performed. At the conclusion of the experiments, *in vivo* association of these factors and Smad4 on the SBE of the claudin-1 promoter will be examined by ChIP assays using respective antibodies.

Determine whether BMP signaling plays a role in EMT reversal in Smad4 expressing SW480 cells

Rationale: Smad4 expression in SW480 cells resulted in down-regulation of claudin-1

independently of TGF-β signaling and an increase in BMP signaling. BMP signaling has been implicated in reversal of EMTEMT [179].

We will determine whether blocking autocrine BMP signaling using recombinant Noggin protein [12] or BMP neutralizing antibody can 1) block BMP-driven transcription of a BMP reporter construct and 2) inhibit the downstream effects caused by Smad4 in SW480 cells, including reversal of EMT, suppression of claudin-1 and re-expression of E-cadherin.

REFERENCES

1. Feng, X.H. and R. Derynck, *Specificity and versatility in tgf-beta signaling through Smads.* Annu Rev Cell Dev Biol, 2005. **21**: p. 659-93.

2. Shi, Y. and J. Massague, *Mechanisms of TGF-beta signaling from cell membrane to the nucleus.* Cell, 2003. **113**(6): p. 685-700.

3. Pelton, R., et al., *Immunohistochemical localization of TGF beta 1, TGF beta 2, and TGF beta 3 in the mouse embryo: expression patterns suggest multiple roles during embryonic development.* J. Cell Biol., 1991. **115**(4): p. 1091-1105.

4. Derynck, R., et al., *Synthesis of messenger RNAs for transforming growth factors alpha and beta and the epidermal growth factor receptor by human tumors.* Cancer Res, 1987. **47**(3): p. 707-12.

5. Dickson, R.B., et al., *Activation of growth factor secretion in tumorigenic states of breast cancer induced by 17 beta-estradiol or v-Ha-ras oncogene.* Proc Natl Acad Sci U S A, 1987. **84**(3): p. 837-41.

6. Bottinger, E.P., et al., *The recombinant proregion of transforming growth factor beta1 (latency-associated peptide) inhibits active transforming growth factor beta1 in transgenic mice.* Proc Natl Acad Sci U S A, 1996. **93**(12): p. 5877-82.

7. Kojima, S., et al., *Transcriptional activation of urokinase by the Kruppel-like factor Zf9/COPEB activates latent TGF-beta 1 in vascular endothelial cells.* Blood, 2000. **95**(4): p. 1309-1316.

8. Khalil, N., et al., *Plasmin regulates the activation of cell-associated latent TGF-beta 1 secreted by rat alveolar macrophages after in vivo bleomycin injury.* Am J Respir Cell Mol Biol, 1996. **15**(2): p. 252-9.

9. Massague, J. and Y.G. Chen, *Controlling TGF-beta signaling.* Genes Dev, 2000. **14**(6): p. 627-44.

10. de Winter, J.P., et al., *Follistatins neutralize activin bioactivity by inhibition of activin binding to its type II receptors.* Mol Cell Endocrinol, 1996. **116**(1): p. 105-14.

11. Iemura, S., et al., *Direct binding of follistatin to a complex of bone-morphogenetic protein and its receptor inhibits ventral and epidermal cell fates in early Xenopus embryo.* Proc Natl Acad Sci U S A, 1998. **95**(16): p. 9337-42.

12. Groppe, J., et al., *Structural basis of BMP signalling inhibition by the cystine knot protein Noggin.* Nature, 2002. **420**(6916): p. 636-42.

13. Piccolo, S., et al., *Dorsoventral patterning in Xenopus: inhibition of ventral signals by direct binding of chordin to BMP-4.* Cell, 1996. **86**(4): p. 589-98.

14. Feng, X.H. and R. Derynck, *SPECIFICITY AND VERSATILITY IN TGF-beta SIGNALING THROUGH SMADS.* Annu Rev Cell Dev Biol, 2005. **21**: p. 659-693.

15. Massague, J., *TGF-beta signal transduction.* Annu Rev Biochem, 1998. **67**: p. 753-91.

16. Derynck, R. and Y.E. Zhang, *Smad-dependent and Smad-independent pathways in TGF-[beta] family signalling.* 2003. **425**(6958): p. 577-584.

17. Mulder, K.M., *Role of Ras and Mapks in TGF[beta] signaling.* Cytokine & Growth Factor Reviews, 2000. **11**(1-2): p. 23-35.

18. Laiho, M., et al., *Growth inhibition by TGF-beta linked to suppression of retinoblastoma protein phosphorylation.* Cell, 1990. **62**(1): p. 175-85.

19. Moses, H.L., E.Y. Yang, and J.A. Pietenpol, *TGF-beta stimulation and inhibition of cell proliferation: new mechanistic insights.* Cell, 1990. **63**(2): p. 245-7.

20. Pietenpol, J.A., et al., *TGF-beta 1 inhibition of c-myc transcription and growth in keratinocytes is abrogated by viral transforming proteins with pRB binding domains.* Cell, 1990. **61**(5): p. 777-85.

21. Oberhammer, F.A., et al., *Induction of apoptosis in cultured hepatocytes and in regressing liver by transforming growth factor beta 1.* Proc Natl Acad Sci U S A, 1992. **89**(12): p. 5408-12.

22. Elliott, R.L. and G.C. Blobe, *Role of transforming growth factor Beta in human cancer.* J Clin Oncol, 2005. **23**(9): p. 2078-93.

23. Turco, A., et al., *Overexpression of transforming growth factor beta-type II receptor reduces tumorigenicity and metastastic potential of K-ras-transformed thyroid cells.* Int J Cancer, 1999. **80**(1): p. 85-91.

24. Bottinger, E.P., et al., *Transgenic mice overexpressing a dominant-negative mutant type II transforming growth factor beta receptor show enhanced tumorigenesis in the mammary gland and lung in response to the carcinogen 7,12-dimethylbenz-[a]-anthracene.* Cancer Res, 1997. **57**(24): p. 5564-70.

25. Hannon, G.J. and D. Beach, *p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest.* Nature, 1994. **371**(6494): p. 257-61.

26. Datto, M.B., et al., *Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism.* Proc Natl Acad Sci U S A, 1995. **92**(12): p. 5545-9.

27. Reynisdottir, I., et al., *Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-beta.* Genes Dev, 1995. **9**(15): p. 1831-45.

28. Sandhu, C., et al., *Transforming growth factor beta stabilizes p15INK4B protein, increases p15INK4B-cdk4 complexes, and inhibits cyclin D1-cdk4 association in human mammary epithelial cells.* Mol Cell Biol, 1997. **17**(5): p. 2458-67.

29. Coffey, R.J., Jr., et al., *Selective inhibition of growth-related gene expression in murine keratinocytes by transforming growth factor beta.* Mol Cell Biol, 1988. **8**(8): p. 3088-93.

30. Ewen, M.E., et al., *p53-dependent repression of CDK4 translation in TGF-beta-induced G1 cell-cycle arrest.* Genes Dev, 1995. **9**(2): p. 204-17.

31. Derynck, R., R.J. Akhurst, and A. Balmain, *TGF-[beta] signaling in tumor suppression and cancer progression.* 2001. **29**(2): p. 117-129.

32. Mithani, S.K., et al., *Smad3 has a critical role in TGF-beta-mediated growth inhibition and apoptosis in colonic epithelial cells.* J Surg Res, 2004. **117**(2): p. 296-305.

33. Yamamura, Y., et al., *Critical role of Smads and AP-1 complex in transforming growth factor-beta -dependent apoptosis.* J Biol Chem, 2000. **275**(46): p. 36295-302.

34. Landstrom, M., et al., *Smad7 mediates apoptosis induced by transforming growth factor beta in prostatic carcinoma cells.* Curr Biol, 2000. **10**(9): p. 535-8.

35. Perlman, R., et al., *TGF-beta-induced apoptosis is mediated by the adapter protein Daxx that facilitates JNK activation.* Nat Cell Biol, 2001. **3**(8): p. 708-14.

36. Saltzman, A., et al., *Transforming growth factor-beta-mediated apoptosis in the Ramos B-lymphoma cell line is accompanied by caspase activation and Bcl-XL downregulation.* Exp Cell Res, 1998. **242**(1): p. 244-54.

37. Chen, R.H. and T.Y. Chang, *Involvement of caspase family proteases in transforming growth factor-beta-induced apoptosis.* Cell Growth Differ, 1997. **8**(7): p. 821-7.

38. Ellenrieder, V., et al., *TGF-beta-induced invasiveness of pancreatic cancer cells is mediated by matrix metalloproteinase-2 and the urokinase plasminogen activator system.* Int J Cancer, 2001. **93**(2): p. 204-11.

39. Lee, J., et al., *TGF-beta1 inhibition of apoptosis through the transcriptional up-regulation of Bcl-X(L) in human monocytic leukemia U937 cells.* Exp Mol Med, 1999. **31**(3): p. 126-33.

40. Lei, X., et al., *Autocrine TGFbeta supports growth and survival of human breast cancer MDA-MB-231 cells.* Oncogene, 2002. **21**(49): p. 7514-23.

41. Miettinen, P., et al., *TGF-beta induced transdifferentiation of mammary epithelial cells to mesenchymal cells: involvement of type I receptors.* J. Cell Biol., 1994. **127**(6): p. 2021-2036.

42. Donovan, D., et al., *TGF beta-1 regulation of VEGF production by breast cancer cells.* Ann Surg Oncol, 1997. **4**(8): p. 621-7.

43. Hirashima, Y., et al., *Transforming Growth Factor-{beta}1 Produced by Ovarian Cancer Cell Line HRA Stimulates Attachment and Invasion through an Up-regulation of Plasminogen Activator Inhibitor Type-1 in Human Peritoneal Mesothelial Cells.* J. Biol. Chem., 2003. **278**(29): p. 26793-26802.

44. Huang, F., et al., *Transforming growth factor beta 1 (TGF beta 1) is an autocrine positive regulator of colon carcinoma U9 cells in vivo as shown by transfection of a TGF beta 1 antisense expression plasmid.* Cell Growth Differ, 1995. **6**(12): p. 1635-1642.

45. Kakonen, S.M., et al., *Transforming growth factor-beta stimulates parathyroid hormone-related protein and osteolytic metastases via Smad and mitogen-activated protein kinase signaling pathways.* J Biol Chem, 2002. **277**(27): p. 24571-8.

46. Thiery, J.P., *Epithelial-mesenchymal transitions in development and pathologies.* Curr Opin Cell Biol, 2003. **15**(6): p. 740-6.

47. Cui, W., et al., *TGFbeta1 inhibits the formation of benign skin tumors, but enhances progression to invasive spindle carcinomas in transgenic mice.* Cell, 1996. **86**(4): p. 531-42.

48. Oft, M., et al., *TGF-beta1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells.* Genes Dev, 1996. **10**(19): p. 2462-77.

49. Oft, M., K.-H. Heider, and H. Beug, *TGF[beta] signaling is necessary for carcinoma cell invasiveness and metastasis.* Current Biology, 1998. **8**(23): p. 1243-1252.

50. Valdes, F., et al., *The epithelial mesenchymal transition confers resistance to the apoptotic effects of transforming growth factor Beta in fetal rat hepatocytes.* Mol Cancer Res, 2002. **1**(1): p. 68-78.

51. Farina, A.R., et al., *Transforming growth factor-beta1 enhances the invasiveness of human MDA-MB-231 breast cancer cells by up-regulating urokinase activity.* Int J Cancer, 1998. **75**(5): p. 721-30.

52. Fujimoto, K., et al., *Transforming growth factor-beta1 promotes invasiveness after cellular transformation with activated Ras in intestinal epithelial cells.* Exp Cell Res, 2001. **266**(2): p. 239-49.

53. McEarchern, J.A., et al., *Invasion and metastasis of a mammary tumor involves TGF-beta signaling.* Int J Cancer, 2001. **91**(1): p. 76-82.

54. Yang, Y.A., et al., *Lifetime exposure to a soluble TGF-beta antagonist protects mice against metastasis without adverse side effects.* J Clin Invest, 2002. **109**(12): p. 1607-15.

55. Park, B.J., et al., *Mitogenic conversion of transforming growth factor-beta1 effect by oncogenic Ha-Ras-induced activation of the mitogen-activated protein kinase signaling pathway in human prostate cancer.* Cancer Res, 2000. **60**(11): p. 3031-8.

56. Shih, S.C. and K.P. Claffey, *Role of AP-1 and HIF-1 transcription factors in TGF-beta activation of VEGF expression.* Growth Factors, 2001. **19**(1): p. 19-34.

57. Teraoka, H., et al., *Enhanced VEGF production and decreased immunogenicity induced by TGF-beta 1 promote liver metastasis of pancreatic cancer.* Br J Cancer, 2001. **85**(4): p. 612-7.

58. Kirkbride, K.C. and G.C. Blobe, *Inhibiting the TGF-beta signalling pathway as a means of cancer immunotherapy.* Expert Opin Biol Ther, 2003. **3**(2): p. 251-61.

59. Torre-Amione, G., et al., *A highly immunogenic tumor transfected with a murine transforming growth factor type beta 1 cDNA escapes immune surveillance.* Proc Natl Acad Sci U S A, 1990. **87**(4): p. 1486-90.

60. Leach, D.R., M.F. Krummel, and J.P. Allison, *Enhancement of antitumor immunity by CTLA-4 blockade.* Science, 1996. **271**(5256): p. 1734-6.

61. Peled, A., et al., *Interactions between leukemia cells and bone marrow stromal cells: stroma-supported growth vs. serum dependence and the roles of TGF-beta and M-CSF.* Exp Hematol, 1996. **24**(6): p. 728-37.

62. Bakin, A.V., et al., *Phosphatidylinositol 3-Kinase Function Is Required for Transforming Growth Factor beta -mediated Epithelial to Mesenchymal Transition and Cell Migration.* J. Biol. Chem., 2000. **275**(47): p. 36803-36810.

63. Sun, L. and C. Chen, *Expression of transforming growth factor beta type III receptor suppresses tumorigenicity of human breast cancer MDA-MB-231 cells.* J Biol Chem, 1997. **272**(40): p. 25367-72.

64. Carmeliet, P., et al., *Urokinase-generated plasmin activates matrix metalloproteinases during aneurysm formation.* Nat Genet, 1997. **17**(4): p. 439-44.

65. Duffy, M.J., et al., *Urokinase-plasminogen activator, a marker for aggressive breast carcinomas. Preliminary report.* Cancer, 1988. **62**(3): p. 531-3.

66. Duffy, M.J., et al., *Urokinase plasminogen activator: a prognostic marker in multiple types of cancer.* J Surg Oncol, 1999. **71**(2): p. 130-5.

67. Agrotis, A., N. Kalinina, and A. Bobik, *Transforming growth factor-beta, cell signaling and cardiovascular disorders.* Curr Vasc Pharmacol, 2005. **3**(1): p. 55-61.

68. Huang, S.S. and J.S. Huang, *TGF-beta control of cell proliferation.* J Cell Biochem, 2005. **96**(3): p. 447-62.

69. Mead, A.L., et al., *Evaluation of anti-TGF-beta2 antibody as a new postoperative anti-scarring agent in glaucoma surgery.* Invest Ophthalmol Vis Sci, 2003. **44**(8): p. 3394-401.

70. Cordeiro, M.F., *Technology evaluation: lerdelimumab, Cambridge Antibody Technology.* Curr Opin Mol Ther, 2003. **5**(2): p. 199-203.

71. Schlingensiepen, K.H., et al., *Targeted tumor therapy with the TGF-beta2 antisense compound AP 12009.* Cytokine Growth Factor Rev, 2006. **17**(1-2): p. 129-39.

72. Yang, D.C., R.L. Elliott, and J.F. Head, *Gene targets of antisense therapies in breast cancer.* Expert Opin Ther Targets, 2002. **6**(3): p. 375-85.

73. Singh, J., et al., *Transforming the TGFbeta pathway: convergence of distinct lead generation strategies on a novel kinase pharmacophore for TbetaRI (ALK5).* Curr Opin Drug Discov Devel, 2004. **7**(4): p. 437-45.

74. Peng, S.B., et al., *Kinetic characterization of novel pyrazole TGF-beta receptor I kinase inhibitors and their blockade of the epithelial-mesenchymal transition.* Biochemistry, 2005. **44**(7): p. 2293-304.

75. Huntley, S.P., et al., *Attenuated type II TGF-beta receptor signalling in human malignant oral keratinocytes induces a less differentiated and more aggressive phenotype that is associated with metastatic dissemination.* Int J Cancer, 2004. **110**(2): p. 170-6.

76. Jonk, L.J.C., et al., *Identification and Functional Characterization of a Smad Binding Element (SBE) in the JunB Promoter That Acts as a Transforming Growth Factor-beta , Activin, and Bone Morphogenetic Protein-inducible Enhancer.* J. Biol. Chem., 1998. **273**(33): p. 21145-21152.

77. Ishida, W., et al., *Smad6 is a Smad1/5-induced smad inhibitor. Characterization of bone morphogenetic protein-responsive element in the mouse Smad6 promoter.* J Biol Chem, 2000. **275**(9): p. 6075-9.

78. Kim, J., et al., *Drosophila Mad binds to DNA and directly mediates activation of vestigial by Decapentaplegic.* Nature, 1997. **388**(6639): p. 304-8.

79. Janknecht, R., N.J. Wells, and T. Hunter, *TGF-beta-stimulated cooperation of smad proteins with the coactivators CBP/p300.* Genes Dev, 1998. **12**(14): p. 2114-9.

80. Bai, R.Y., et al., *SMIF, a Smad4-interacting protein that functions as a co-activator in TGFbeta signalling.* Nat Cell Biol, 2002. **4**(3): p. 181-90.

81. Luo, K., et al., *The Ski oncoprotein interacts with the Smad proteins to repress TGFbeta signaling.* Genes Dev., 1999. **13**(17): p. 2196-2206.

82. Hahn, S.A., et al., *DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1.* Science, 1996. **271**(5247): p. 350-3.

83. Alazzouzi, H., et al., *SMAD4 as a prognostic marker in colorectal cancer.* Clin Cancer Res, 2005. **11**(7): p. 2606-11.

84. Miyaki, M., et al., *Higher frequency of Smad4 gene mutation in human colorectal cancer with distant metastasis.* Oncogene, 1999. **18**(20): p. 3098-103.

85. Muller, N., et al., *Smad4 induces the tumor suppressor E-cadherin and P-cadherin in colon carcinoma cells.* Oncogene, 2002. **21**(39): p. 6049-58.

86. Reinacher-Schick, A., et al., *Loss of Smad4 correlates with loss of the invasion suppressor E-cadherin in advanced colorectal carcinomas.* J Pathol, 2004. **202**(4): p. 412-20.

87. Schwarte-Waldhoff, I., et al., *DPC4/SMAD4 mediated tumor suppression of colon carcinoma cells is associated with reduced urokinase expression.* Oncogene, 1999. **18**(20): p. 3152-8.

88. Schwarte-Waldhoff, I., et al., *Smad4/DPC4-mediated tumor suppression through suppression of angiogenesis.* Proc Natl Acad Sci U S A, 2000. **97**(17): p. 9624-9.

89. Valcourt, U., et al., *TGF-beta and the Smad signaling pathway support transcriptomic reprogramming during epithelial-mesenchymal cell transition.* Mol Biol Cell, 2005. **16**(4): p. 1987-2002.

90. Plouet, J., et al., *Extracellular cleavage of the vascular endothelial growth factor 189-amino acid form by urokinase is required for its mitogenic effect.* J Biol Chem, 1997. **272**(20): p. 13390-6.

91. Stepanova, V.V. and V.A. Tkachuk, *Urokinase as a multidomain protein and polyfunctional cell regulator.* Biochemistry (Mosc), 2002. **67**(1): p. 109-18.

92. McColl, B.K., et al., *Plasmin activates the lymphangiogenic growth factors VEGF-C and VEGF-D.* J Exp Med, 2003. **198**(6): p. 863-8.

93. Odekon, L.E., F. Blasi, and D.B. Rifkin, *Requirement for receptor-bound urokinase in plasmin-dependent cellular conversion of latent TGF-beta to TGF-beta.* J Cell Physiol, 1994. **158**(3): p. 398-407.

94. Bizik, J., et al., *Active transforming growth factor-beta in human melanoma cell lines: no evidence for plasmin-related activation of latent TGF-beta.* J Cell Biochem, 1996. **62**(1): p. 113-22.

95. Konakova, M., F. Hucho, and W.D. Schleuning, *Downstream targets of urokinase-type plasminogen-activator-mediated signal transduction.* Eur J Biochem, 1998. **253**(2): p. 421-9.

96. Ahmed, N., et al., *Downregulation of urokinase plasminogen activator receptor expression inhibits Erk signalling with concomitant suppression of invasiveness due to loss of uPAR-beta1 integrin complex in colon cancer cells.* Br J Cancer, 2003. **89**(2): p. 374-84.

97. Busso, N., et al., *Induction of cell migration by pro-urokinase binding to its receptor: possible mechanism for signal transduction in human epithelial cells.* J Cell Biol, 1994. **126**(1): p. 259-70.

98. Odekon, L.E., Y. Sato, and D.B. Rifkin, *Urokinase-type plasminogen activator mediates basic fibroblast growth factor-induced bovine endothelial cell migration independent of its proteolytic activity.* J Cell Physiol, 1992. **150**(2): p. 258-63.

99. Andreasen, P.A., et al., *The urokinase-type plasminogen activator system in cancer metastasis: a review.* Int J Cancer, 1997. **72**(1): p. 1-22.

100. Nobuhara, M., et al., *A comparative study of high molecular weight urokinase and low molecular weight urokinase.* J Biochem (Tokyo), 1981. **90**(1): p. 225-32.

101. Takasugi, S., et al., *Detection of low molecular weight urokinase in plasma of patient with acute pancreatitis followed by disseminated intravascular coagulation.* Hiroshima J Med Sci, 1984. **33**(2): p. 265-9.

102. Barlow, G.H., C.W. Francis, and V.J. Marder, *On the conversion of high molecular weight urokinase to the low molecular weight form by plasmin.* Thromb Res, 1981. **23**(6): p. 541-7.

103. Pannell, R. and V. Gurewich, *Activation of plasminogen by single-chain urokinase or by two-chain urokinase--a demonstration that single-chain urokinase has a low catalytic activity (pro-urokinase).* Blood, 1987. **69**(1): p. 22-6.

104. Paysant, J., et al., *Regulation of the uPAR/uPA system expressed on monocytes by the deactivating cytokines, IL-4, IL-10 and IL-13: consequences on cell adhesion to vitronectin and fibrinogen.* Br J Haematol, 1998. **100**(1): p. 45-51.

105. Mimuro, J., et al., *Reversible interactions between plasminogen activators and plasminogen activator inhibitor-1.* Biochim Biophys Acta, 1992. **1160**(3): p. 325-34.

106. Cubellis, M.V., T.C. Wun, and F. Blasi, *Receptor-mediated internalization and degradation of urokinase is caused by its specific inhibitor PAI-1.* Embo J, 1990. **9**(4): p. 1079-85.

107. Nykjaer, A., et al., *Recycling of the urokinase receptor upon internalization of the uPA:serpin complexes.* Embo J, 1997. **16**(10): p. 2610-20.

108. Schmitt, M., et al., *Clinical impact of the plasminogen activation system in tumor invasion and metastasis: prognostic relevance and target for therapy.* Thromb Haemost, 1997. **78**(1): p. 285-96.

109. Foekens, J.A., et al., *The Urokinase System of Plasminogen Activation and Prognosis in 2780 Breast Cancer Patients.* Cancer Res, 2000. **60**(3): p. 636-643.

110. Sidenius, N. and F. Blasi, *The urokinase plasminogen activator system in cancer: recent advances and implication for prognosis and therapy.* Cancer Metastasis Rev, 2003. **22**(2-3): p. 205-22.

111. Tanaka, Y., et al., *Transforming growth factor-beta1-dependent urokinase up-regulation and promotion of invasion are involved in Src-MAPK-dependent signaling in human ovarian cancer cells.* J Biol Chem, 2004. **279**(10): p. 8567-76.

112. Yu, H.R. and R.M. Schultz, *Relationship between secreted urokinase plasminogen activator activity and metastatic potential in murine B16 cells transfected with human urokinase sense and antisense genes.* Cancer Res, 1990. **50**(23): p. 7623-33.

113. Ossowski, L. and E. Reich, *Antibodies to plasminogen activator inhibit human tumor metastasis.* Cell, 1983. **35**(3 Pt 2): p. 611-9.

114. Nozaki, S., et al., *Inhibition of invasion and metastasis in oral cancer by targeting urokinase-type plasminogen activator receptor.* Oral Oncol, 2005. **41**(10): p. 971-7.

115. Setyono-Han, B., et al., *Suppression of rat breast cancer metastasis and reduction of primary tumour growth by the small synthetic urokinase inhibitor WX-UK1.* Thromb Haemost, 2005. **93**(4): p. 779-86.

116. Lakka, S.S., et al., *Adenovirus-mediated antisense urokinase-type plasminogen activator receptor gene transfer reduces tumor cell invasion and metastasis in non-small cell lung cancer cell lines.* Clin Cancer Res, 2001. **7**(4): p. 1087-93.

117. Zhu, F., et al., *cDNA transfection of amino-terminal fragment of urokinase efficiently inhibits cancer cell invasion and metastasis.* DNA Cell Biol, 2001. **20**(5): p. 297-305.

118. Knust, E. and O. Bossinger, *Composition and formation of intercellular junctions in epithelial cells.* Science, 2002. **298**(5600): p. 1955-9.

119. Al Moustafa, A.E., et al., *Identification of genes associated with head and neck carcinogenesis by cDNA microarray comparison between matched primary normal epithelial and squamous carcinoma cells.* Oncogene, 2002. **21**(17): p. 2634-40.

120. Kominsky, S.L., et al., *Loss of the tight junction protein claudin-7 correlates with histological grade in both ductal carcinoma in situ and invasive ductal carcinoma of the breast.* Oncogene, 2003. **22**(13): p. 2021-33.

121. Rendon-Huerta, E., et al., *Comparison of three integral tight junction barrier proteins in Barrett's epithelium versus normal esophageal epithelium.* Am J Gastroenterol, 2003. **98**(8): p. 1901-3.

122. Rangel, L.B., et al., *Tight junction proteins claudin-3 and claudin-4 are frequently overexpressed in ovarian cancer but not in ovarian cystadenomas.* Clin Cancer Res, 2003. **9**(7): p. 2567-75.

123. Nichols, L.S., R. Ashfaq, and C.A. Iacobuzio-Donahue, *Claudin 4 protein expression in primary and metastatic pancreatic cancer: support for use as a therapeutic target.* Am J Clin Pathol, 2004. **121**(2): p. 226-30.

124. Miwa, N., et al., *Involvement of claudin-1 in the beta-catenin/Tcf signaling pathway and its frequent upregulation in human colorectal cancers.* Oncol Res, 2000. **12**(11-12): p. 469-76.

125. Dhawan, P., et al., *Claudin-1 regulates cellular transformation and metastatic behavior in colon cancer.* J Clin Invest, 2005. **115**(7): p. 1765-1776.

126. Cano, A., et al., *The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression.* Nat Cell Biol, 2000. **2**(2): p. 76-83.

127. Batlle, E., et al., *The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells.* Nat Cell Biol, 2000. **2**(2): p. 84-9.

128. Martinez-Estrada, O.M., et al., *The transcription factors Slug and Snail act as repressors of Claudin-1 expression in epithelial cells.* Biochem J, 2005.

129. Korinek, V., et al., *Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma.* Science, 1997. **275**(5307): p. 1784-7.

130. Kolligs, F.T., G. Bommer, and B. Goke, *Wnt/beta-catenin/tcf signaling: a critical pathway in gastrointestinal tumorigenesis.* Digestion, 2002. **66**(3): p. 131-44.

131. Suzuki, Y., et al., *Physical interaction between retinoic acid receptor and Sp1: mechanism for induction of urokinase by retinoic acid.* Blood, 1999. **93**(12): p. 4264-76.

132. Wrana, J.L., et al., *TGF beta signals through a heteromeric protein kinase receptor complex.* Cell, 1992. **71**(6): p. 1003-14.

133. Mignatti, P., E. Robbins, and D.B. Rifkin, *Tumor invasion through the human amniotic membrane: requirement for a proteinase cascade.* Cell, 1986. **47**(4): p. 487-98.

134. Zavadil, J. and E.P. Bottinger, *TGF-beta and epithelial-to-mesenchymal transitions.* Oncogene, 2005. **24**(37): p. 5764-74.

135. Lin, H.Y., et al., *Expression cloning of the TGF-beta type II receptor, a functional transmembrane serine/threonine kinase.* Cell, 1992. **68**(4): p. 775-85.

136. Wrana, J.L., et al., *Mechanism of activation of the TGF-beta receptor.* Nature, 1994. **370**(6488): p. 341-7.

137. Frey, R. and K. Mulder, *Involvement of extracellular signal-regulated kinase 2 and stress-activated protein kinase/Jun N-terminal kinase activation by transforming growth factor beta in the negative growth control of breast cancer cells.* Cancer Res, 1997. **57**(4): p. 628-633.

138. Bakin, A.V., et al., *p38 mitogen-activated protein kinase is required for TGF{beta}-mediated fibroblastic transdifferentiation and cell migration.* J Cell Sci, 2002. **115**(15): p. 3193-3206.

139. Bhowmick, N.A., et al., *Integrin beta 1 Signaling Is Necessary for Transforming Growth Factor-beta Activation of p38MAPK and Epithelial Plasticity.* J. Biol. Chem., 2001. **276**(50): p. 46707-46713.

140. Andreasen, P.A., R. Egelund, and H.H. Petersen, *The plasminogen activation system in tumor growth, invasion, and metastasis.* Cell Mol Life Sci, 2000. **57**(1): p. 25-40.

141. Reuning, U., et al., *Multifunctional potential of the plasminogen activation system in tumor invasion and metastasis (review).* Int J Oncol, 1998. **13**(5): p. 893-906.

142. Frandsen, T.L., et al., *Direct evidence of the importance of stromal urokinase plasminogen activator (uPA) in the growth of an experimental human breast cancer*

using a combined uPA gene-disrupted and immunodeficient xenograft model. Cancer Res, 2001. **61**(2): p. 532-7.

143. Martinez-Carpio, P.A., et al., *Constitutive and regulated secretion of epidermal growth factor and transforming growth factor-beta1 in MDA-MB-231 breast cancer cell line in 11-day cultures.* Cell Signal, 1999. **11**(10): p. 753-7.

144. Martinez-Carpio, P.A., et al., *Secretion and dual regulation between epidermal growth factor and transforming growth factor-beta1 in MDA-MB-231 cell line in 42-hour-long cultures.* Cancer Lett, 1999. **147**(1-2): p. 25-9.

145. Chen, C.-R., Y. Kang, and J. Massague, *Inaugural Article: Defective repression of c-myc in breast cancer cells: A loss at the core of the transforming growth factor beta growth arrest program.* PNAS, 2001. **98**(3): p. 992-999.

146. Matrisian, L.M., *Cancer biology: extracellular proteinases in malignancy.* Curr Biol, 1999. **9**(20): p. R776-8.

147. Matrisian, L.M., et al., *Matrix-degrading metalloproteinases in tumor progression.* Princess Takamatsu Symp, 1994. **24**: p. 152-61.

148. Hocevar, B.A., T.L. Brown, and P.H. Howe, *TGF-beta induces fibronectin synthesis through a c-Jun N-terminal kinase-dependent, Smad4-independent pathway.* Embo J, 1999. **18**(5): p. 1345-56.

149. Chen, R.H., R. Ebner, and R. Derynck, *Inactivation of the type II receptor reveals two receptor pathways for the diverse TGF-beta activities.* Science, 1993. **260**(5112): p. 1335-8.

150. Wun, T. and E. Reich, *An inhibitor of plasminogen activation from human placenta. Purification and characterization.* J. Biol. Chem., 1987. **262**(8): p. 3646-3653.

151. Westerhausen, D., Jr, W. Hopkins, and J. Billadello, *Multiple transforming growth factor-beta-inducible elements regulate expression of the plasminogen activator inhibitor type-1 gene in Hep G2 cells.* J. Biol. Chem., 1991. **266**(2): p. 1092-1100.

152. D'Orazio, D., et al., *Cooperation of two PEA3/AP1 sites in uPA gene induction by TPA and FGF-2.* Gene, 1997. **201**(1-2): p. 179-87.

153. Moses, H.L., et al., *TGF beta regulation of epithelial cell proliferation: role of tumor suppressor genes.* Princess Takamatsu Symp, 1991. **22**: p. 183-95.

154. Tang, B., et al., *Transforming growth factor-beta1 is a new form of tumor suppressor with true haploid insufficiency.* Nat Med, 1998. **4**(7): p. 802-7.

155. Caulin, C., et al., *Chronic exposure of cultured transformed mouse epidermal cells to transforming growth factor-beta 1 induces an epithelial-mesenchymal transdifferentiation and a spindle tumoral phenotype.* Cell Growth Differ, 1995. **6**(8): p. 1027-35.

156. Roman, C.D., et al., *Induction of cyclooxygenase-2 and invasiveness by transforming growth factor-beta(1) in immortalized mouse colonocytes expressing oncogenic Ras.* J Gastrointest Surg, 2002. **6**(3): p. 304-9.

157. Huang, F., et al., *Transforming growth factor beta 1 (TGF beta 1) is an autocrine positive regulator of colon carcinoma U9 cells in vivo as shown by transfection of a TGF beta 1 antisense expression plasmid*
Cell Growth Differ, 1995. **6**(12): p. 1635-1642.

158. Dean, J.L., et al., *The 3' untranslated region of tumor necrosis factor alpha mRNA is a target of the mRNA-stabilizing factor HuR.* Mol Cell Biol, 2001. **21**(3): p. 721-30.

159. Sheng, H., et al., *Transforming growth factor-beta1 enhances Ha-ras-induced expression of cyclooxygenase-2 in intestinal epithelial cells via stabilization of mRNA.* J Biol Chem, 2000. **275**(9): p. 6628-35.

160. Zubiaga, A.M., J.G. Belasco, and M.E. Greenberg, *The nonamer UUAUUUAUU is the key AU-rich sequence motif that mediates mRNA degradation.* Mol Cell Biol, 1995. **15**(4): p. 2219-30.

161. Kozma, S.C., et al., *The human c-Kirsten ras gene is activated by a novel mutation in codon 13 in the breast carcinoma cell line MDA-MB231.* Nucleic Acids Res, 1987. **15**(15): p. 5963-71.

162. Nanbu, R., et al., *Enhanced stability of urokinase-type plasminogen activator mRNA in metastatic breast cancer MDA-MB-231 cells and LLC-PK1 cells down-regulated for protein kinase C--correlation with cytoplasmic heterogeneous nuclear ribonucleoprotein C.* Eur J Biochem, 1997. **247**(1): p. 169-74.

163. Higazi, A., et al., *Single-chain urokinase-type plasminogen activator bound to its receptor is relatively resistant to plasminogen activator inhibitor type 1.* Blood, 1996. **87**(9): p. 3545-3549.

164. Farina, A.R., et al., *Identification of plasminogen in Matrigel and its activation by reconstitution of this basement membrane extract.* Biotechniques, 1996. **21**(5): p. 904-9.

165. Baricos, W.H., et al., *ECM degradation by cultured human mesangial cells is mediated by a PA/plasmin/MMP-2 cascade.* Kidney Int, 1995. **47**(4): p. 1039-47.

166. Baramova, E.N., et al., *Involvement of PA/plasmin system in the processing of pro-MMP-9 and in the second step of pro-MMP-2 activation.* FEBS Lett, 1997. **405**(2): p. 157-62.

167. Ramos-DeSimone, N., et al., *Activation of matrix metalloproteinase-9 (MMP-9) via a converging plasmin/stromelysin-1 cascade enhances tumor cell invasion.* J Biol Chem, 1999. **274**(19): p. 13066-76.

168. Lawrence, D.A., R. Pircher, and P. Jullien, *Conversion of a high molecular weight latent beta-TGF from chicken embryo fibroblasts into a low molecular weight active beta-TGF under acidic conditions.* Biochem Biophys Res Commun, 1985. **133**(3): p. 1026-34.

169. Vrana, J.A., et al., *Expression of tissue factor in tumor stroma correlates with progression to invasive human breast cancer: paracrine regulation by carcinoma cell-derived members of the transforming growth factor beta family.* Cancer Res, 1996. **56**(21): p. 5063-70.

170. Walker, R.A., S.J. Dearing, and B. Gallacher, *Relationship of transforming growth factor beta 1 to extracellular matrix and stromal infiltrates in invasive breast carcinoma.* Br J Cancer, 1994. **69**(6): p. 1160-5.

171. Fisher, J.L., et al., *Urokinase plasminogen activator system gene expression is increased in human breast carcinoma and its bone metastases--a comparison of normal breast tissue, non-invasive and invasive carcinoma and osseous metastases.* Breast Cancer Res Treat, 2000. **61**(1): p. 1-12.

172. Janicke, F., M. Schmitt, and H. Graeff, *Clinical relevance of the urokinase-type and tissue-type plasminogen activators and of their type 1 inhibitor in breast cancer.* Semin Thromb Hemost, 1991. **17**(3): p. 303-12.

173. Massague, J., S.W. Blain, and R.S. Lo, *TGFbeta signaling in growth control, cancer, and heritable disorders.* Cell, 2000. **103**(2): p. 295-309.

174. Coffey, R.J., Jr., et al., *Transforming growth factor alpha and beta expression in human colon cancer lines: implications for an autocrine model.* Cancer Res, 1987. **47**(17): p. 4590-4.

175. Jiang, B., et al., *Growth inhibitory signalling by TGF[beta] is blocked in Ras-transformed intestinal epithelial cells at a post-receptor locus.* Cellular Signalling, 2003. **15**(7): p. 699-708.

176. Vermeulen, S.J., et al., *Transition from the noninvasive to the invasive phenotype and loss of alpha-catenin in human colon cancer cells.* Cancer Res, 1995. **55**(20): p. 4722-8.

177. Gottardi, C.J., E. Wong, and B.M. Gumbiner, *E-cadherin suppresses cellular transformation by inhibiting beta-catenin signaling in an adhesion-independent manner.* J Cell Biol, 2001. **153**(5): p. 1049-60.

178. Hewitt, R.E., et al., *Validation of a model of colon cancer progression.* J Pathol, 2000. **192**(4): p. 446-54.

179. Zeisberg, M., et al., *BMP-7 counteracts TGF-beta1-induced epithelial-to-mesenchymal transition and reverses chronic renal injury.* Nat Med, 2003. **9**(7): p. 964-8.

180. Willert, K. and R. Nusse, *[beta]-catenin: a key mediator of Wnt signaling.* Current Opinion in Genetics & Development, 1998. **8**(1): p. 95-102.

181. Kim, D.W. and A.B. Lassar, *Smad-dependent recruitment of a histone deacetylase/Sin3A complex modulates the bone morphogenetic protein-dependent transcriptional repressor activity of Nkx3.2.* Mol Cell Biol, 2003. **23**(23): p. 8704-17.

182. Stroschein, S.L., et al., *Negative Feedback Regulation of TGF- Signaling by the SnoN Oncoprotein.* Science, 1999. **286**(5440): p. 771-774.