Published in final edited form as:

Gastroenterology. 2010 March ; 138(3): 969–980.e3. doi:10.1053/j.gastro.2009.11.004.

Anti-metastatic Role of Smad4 Signaling in Colorectal Cancer

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Abstract

Background & Aims—Transforming growth factor (TGF)- β signaling occurs through Smads 2/3/4, which translocate to the nucleus to regulate transcription; TGF- β has tumor suppressive effects in some tumor models and pro-metastatic effects in others. In patients with colorectal cancer (CRC), mutations or reduced levels of Smad4 have been correlated with reduced survival. However, the function of Smad signaling and the effects of TGF- β receptor kinase inhibitors (TRKI) have not been analyzed during CRC metastasis. We investigated the role of TGF- β /Smad signaling in CRC progression.

Methods—We evaluated the role of TGF- β /Smad signaling on cell proliferation, migration, invasion, tumorigenicity, and metastasis in Smad4-null colon carcinoma cell lines (MC38 and SW620) and in those that transgenically express Smad4. We also determined the effects of a TRKI (LY2109761) in CRC tumor progression and metastasis in mice.

Results—TGF- β induced migration/invasion, tumorigenicity, and metastasis of Smad4-null MC38 and SW620 cells; incubation with LY2109761 reversed these effects. In mice, LY2109761 blocked metastasis of CRC cells to liver, inducing cancer cell expression of E-cadherin and reducing the expression of the tumorigenic proteins MMP-9, nm23, uPA, and COX-2. Transgenic expression of Smad4 significantly reduced the oncogenic potential of MC38 and SW620 cells; in these transgenic cells, TGF- β had tumor suppressor, rather than tumorigenic effects.

Conclusion—TGF- β /Smad signaling suppresses progression and metastasis of CRC cells and tumors in mice. Loss of Smad4 might underlie the functional shift of TGF- β from a tumor suppressor to a tumor promoter; inhibitors of TGF- β signaling might be developed as CRC therapeutics.

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Disclosure: Authors do not have any potential conflict in financial, professional or personal matters.

Authors' contributions

Bixiang Zhang: Doing major experiments, surgical procedures and acquisition of data, analysis and interpretation of data and helping in drafting the manuscript

Sunil K Halder: Acquisition of some data, analysis and interpretation of data, statistical analysis and helping in drafting the manuscript
Nilesh D Kashikar: Acquisition of few data, analysis and interpretation of data, drafting the manuscript, critical revision of the manuscript for important intellectual content

Yong-Jig Cho: Acquisition of few data, analysis and interpretation of data.

Arunima Datta: Acquisition of few data and technical support

David L Gorden: Initiating the surgical procedures and material support

Pran K Datta: Study concept and design, analysis and interpretation of data, drafting of the final manuscript, critical revision of the manuscript for important intellectual content, obtained funding, technical support and study supervision

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Introduction

The loss of TGF- β -induced tumor suppressor function and the gain of tumor promoting effects of TGF- β coupled with increased production of one or more of TGF- β isoforms in advanced cancers play a pivotal role in colorectal cancer (CRC) metastasis. Members of the TGF- β family regulate a wide range of biological processes including cell proliferation, migration, differentiation, apoptosis, and extracellular matrix deposition.¹ Ligand binding to TGF- β receptors (T β RI and T β RII) initiates a Smad2/3/4 complex formation and translocation to the nucleus (Smad pathway) to regulate transcription of target genes. This Smad pathway is important for TGF- β -induced tumor suppressor functions in normal epithelium and in the early stage of tumor progression. To produce the full spectrum of responses, TGF- β can also induce non-Smad signaling pathways like p38MAPK, ERK, PI3K, JNK, or Rho, which are presumably important for pro-oncogenic activities with low levels of input signal.²

Increased production of TGF- β in human tumors can promote tumor growth in an autocrine and/or paracrine manner through the suppression of immunosurveillance, stimulation of connective tissue formation and angiogenesis, and changes that favor invasion and metastasis. Many TGF- β -inducible pro-oncogenic pathways are either independent of Smads, or require cooperation between the Smad and alternative pathways under transforming conditions.¹ Tumors with mutations that completely inactivate T β RII have a better prognosis than those in which the TGF- β signaling pathway remains partially functional. Others and we have shown that the TGF- β receptor kinase inhibitors (TRKI) efficiently attenuate the tumor-promoting effects of TGF- β including EMT, migration, invasion, VEGF secretion and tumorigenicity.³ These inhibitors have been shown to have potential antimetastatic effects in breast⁴, 5, pancreatic⁶ and hepatic metastasis⁷ in animal models. However, little is known about the effect of these antagonists on colorectal cancer metastasis to the liver.

Higher frequency of Smad4 inactivation is observed in liver metastases leading to unfavorable survival.^{8, 9} Colorectal cancer patients with tumors expressing high Smad4 levels have significantly better overall and disease-free survival than patients with low levels.¹⁰ Interestingly, loss of heterozygosity is observed in 95% invasive and metastatic colorectal cancers with Smad4 mutations. In contrast, the Smad pathway has been shown to mediate the pro-metastatic function of TGF- β in breast cancer bone metastasis.¹¹ Blockade of Smad pathway by Smad7 impairs bone and lung metastases.^{12, 13} However, little is known about the mechanism of function of Smad signaling in colorectal cancer liver metastasis and about the role of TRKIs as a therapeutic approach in blocking TGF- β -induced liver metastasis of colorectal cancer especially when Smad4 signaling is absent. Here we have evaluated the protective role of Smad4 in liver metastasis of human and mouse tumor cell lines lacking Smad4. We have also observed that the TRKI, LY2109761 blocks migration, invasion, tumorigenicity and liver metastasis of these cells. Our studies suggest, for the first time, that TRKIs modulate TGF- β -mediated gene responses that facilitate tumor growth and metastasis of colon cancer, and the Smad4 signaling plays a critical anti-metastatic role in CRC.

Materials and Methods

Cell Cultures and Animals

Mouse colon adenocarcinoma cell line, MC38, the engineered MC38 cells expressing firefly luciferase and FET were maintained in DMEM, and human colon adenocarcinoma cell line SW620 (derived from lymph node metastasis) was maintained in Leibovitz's L-15 medium with 2 mM Glutamine and 10 % fetal bovine serum without CO₂. For generating Smad4 stable clones, MC38 and SW620 cell lines were transfected with pCDNA3-Flag-Smad4 or pCDNA3-Flag vector (Invitrogen, Carlsbad, CA) and cells were selected with 600 μ g/ml and 1.2 mg/ml

G418 for MC38 and SW620 cells, respectively. Female C57BL/6 mice (15–16 weeks old) and athymic nude mice (8 weeks old) were used for the experiments.

Reagents and Antibodies

The TRKI (LY2109761) was kindly provided by Dr. Jonathan Yingling (Eli Lilly Pharmaceuticals, Indianapolis, IN).¹⁰ TGF- β 1 was purchased from R&D Systems (Minneapolis, MN). Antibodies were purchased as follows: Zymed Laboratories Inc. (San Francisco, CA): anti-Smad2 and anti-Smad3; Santa Cruz Biotechnology (Santa Cruz, CA): anti-Smad4, anti-Cdk2, anti-Cdk4, anti-Cyclin D1, anti-p21^{Cip1}, anti-MMP9, anti-COX-2, and anti-Rb; BD Biosciences Pharmingen (San Jose, CA): anti-E-cadherin, anti- β -catenin and anti-nm23; Cell Signaling (Denver, MA): anti-phospho-ERK, anti-ERK, anti-phospho-Smad2 and anti-phospho-Rb; and American Diagnostica Inc. (Stamford, CT): anti-uPA.

Immunoprecipitation and Western Blotting

Lysates from TGF- β (5 ng/ml) treated cells were used for immunoprecipitation or western blotting as described previously.¹⁴ Lysates from liver metastases samples were also used for western blot analyses.

Cell Counting Assay

MC38 cells were seeded in 12-well plates and treated with TGF- β 1 (5 ng/ml) and/or LY2109761 (5 μ M) for five days. Cells were counted every day and the average cell numbers from triplicate wells were plotted.

Transcriptional Response Assay

MC38 cells and SW620 clones were seeded into 12-well plate and were transiently co-transfected with CMV- β -gal together with either p3TP-Lux or (CAGA)₉ MLP-Luc in presence or absence of Smad4 expression vector. Transfected cells were treated with 5 ng/ml TGF- β with or without LY2109761 for 22 h. Luciferase activity was normalized to β -galactosidase activity and presented as the mean $\pm$ S.D. of triplicate measurements.

Cell Migration and Invasion Assay

For migration assays, 3×10^4 cells were seeded into the upper chamber of 8- μ M pore transwells and for invasion through either the collagen layer or the matrigel barrier, 3×10^4 cells in 100 μ l of DMEM containing 0.2% BSA were added to the upper chamber of each well. Medium containing 5% FBS, TGF- β (5 ng/ml) and/or LY2109761 (5 μ M) was added to the lower chambers. Cells were allowed to migrate for 5 h for migration assay, for 12 h through collagen and for 21 h through matrigel. Migrated cells were fixed, stained and counted from six random fields and averaged. The experiments were repeated three times.

Wound Healing

Confluent MC38 cells were treated with mitomycin C (0.5 μ g/ml) for four hours prior to wounding. After wounding, cells were treated with TGF- β 1 (5 ng/ml) with or without LY2109761 (5 μ M) for 12 h. Similar assay was performed using SW620 cell clones after treating with TGF- β 1 (5 ng/ml) for 72 h.

In vivo Tumorigenicity Assay

5×10^5 MC38 parental or Smad4 clones and 2×10^6 SW620 clones were subcutaneously injected in one site of C57BL/6 and athymic nude mice (n=5, in each group), respectively. Mice were divided into control (n=5) and LY2109761-treated group (n=5). LY2109761 was administered orally (50 mg/kg) twice a day for 28 days and tumor volumes were calculated by the equation

$V = L \times W^2 \times 0.5$, where L is length and W is width of a tumor. Growth curves for tumors were plotted as the mean volume $\pm$ S.D. of tumors of mice from each group and these experiments were repeated twice.

Immunohistochemical Analysis

Paraffin embedded blocks were prepared from liver metastases and 5 μ m thick serial sections for each slide were fixed with 20% xylene for 20 min, washed with 100% and 95% ethanol for 10 min, and finally washed with water. A standard immunohistochemical method was applied to stain the slides for hematoxylin and eosin (H&E), Tunnel, Phospho-Smad2, E-cadherin, and β -catenin.

Experimental Liver Metastasis Model of Colorectal Cancer

Liver metastases were generated by injecting cells into spleens of wild type syngeneic C57BL/6 mice as described previously.¹⁵ Briefly, spleen was exposed by an incision on the left upper abdomen of mice. Smad4 clones from MC38 (1×10^5) and SW620 cells (5×10^6) in 100 μ l PBS were injected into the spleens of C57BL/6 and athymic nude mice, respectively. Five minutes after injection, spleen was removed, and the abdominal cavity was closed. As MC38 cells are very aggressive, only 1×10^5 cells produce appreciable size liver mets in 3 weeks. SW620 cells are much less aggressive in producing liver/lymph node metastasis. C57BL/6 mice injected with MC38 cells were euthanized after 3 weeks, whereas athymic nude mice injected with SW620 cells were euthanized after eight weeks. Livers were removed, weighed, and examined for metastases. In another experiment, luciferase expressing-MC38 cells were used as described above and mice were treated with LY2109761 (50 mg/Kg) or vehicle orally twice a day until sacrificed. Metastasis was monitored by intraperitoneal injection with 200 μ l of 15 mg/ml Beetle luciferin followed by bioluminescence imaging after 5 min using the IVIS Imaging System (Xenogen). MicroPET (Micro Positron-Emission Tomography) imaging was performed using ^{18}F -2'-fluoro-2'-deoxy-1 β -D-arabionofuranosyl-5-ethyl-uracil (100 μ Ci per animal) intravenously two hours before the procedure. While PET visualizes pathological sites with high contrast, CT scans are able to demonstrate the anatomical details with high resolution. In the same imaging session, CT images were also acquired and the integrated PET/CT scan showed reduction in metastatic foci following treatment with LY2109761. For survival assay, mice were treated with LY2109761 (50 mg/Kg) or vehicle after injecting MC38 cells (1×10^5) into spleens of C57BL/6 mice. Mice were euthanized when evidence of advanced bulky disease was present. The day of sacrifice was considered the day of death for survival evaluation. The mean survival time for each group of mice was determined and the survival was evaluated by log-rank test.

Statistical Analysis

Statistical analyses were performed using Student's t test and SAS 9.1 software for windows (Cary, NC). The Student's t test was used to assess the significance of differences in both in vitro and in vivo studies. Values were considered to be significantly different when P is less than 0.05.

Result

Inactivation of Smad-Dependent Signaling in MC38 Cells due to Loss of Smad4 Expression and Regulation of Non-Smad pathway by TGF- β

To test the role of TGF- β /Smad4 signaling in colorectal cancer metastasis, we first characterized the mouse colon adenocarcinoma cell line (MC38) by testing TGF- β -induced functional complex formation between Smad2/3 and Smad4. TGF- β 1 induced complex formation in FET cells (positive control), whereas neither basal nor TGF- β -induced complex

formation was observed in MC38 cells (Figure 1A). In an attempt to determine why the complex formation is abolished in MC38 cells, we observed appreciable levels of Smad2 and Smad3 in MC38, whereas endogenous Smad4 was undetected (Figure 1A, bottom panels). TGF- β was able to induce phosphorylation of Smad2, but could not induce the expression of PAI-1 and p21^{Cip1}. In an attempt to further test whether the absence of Smad4 in MC38 cells affects downstream signaling, we observed that TGF- β was unable to activate p3TP-Lux and (CAGA)₉ MLP-Luc reporters (Figure 1B). However, re-expression of Smad4 induced both reporter activities in presence and absence of TGF- β , and the TRKI, LY2109761 inhibited TGF- β -induced reporter activities (Figure 1B). The inhibitor reduced phosphorylation of Smad2 in MC38 cells induced by TGF- β (Figure S1). We next determined whether K-Ras mutation in this cell line is playing a role in attenuating TGF- β signaling and did not see any mutations at codon 12, 13 and 61 of K-Ras (data not shown). These results suggest that MC38 cells are non-responsive to TGF- β /Smad signaling due to lack of Smad4 expression.

In addition to Smad signaling, TGF- β can also activate non-Smad pathways such as p38MAPK, ERK, JNK, Akt, RhoA and PP2A etc. We tested the phosphorylation of p38MAPK, ERK, TAK1, JNK and Akt in MC38 and SW620 cells. TGF- β induced phosphorylation of ERK in a time-dependent manner (Figure 1C), and other pathways were unaffected by TGF- β (data not shown). PP2A activity and RhoA activity in these cell lines were not altered by TGF- β (data not shown).

LY2109761 Blocks the Pro-Oncogenic Effects of TGF- β in Smad4-Null MC38 Cells In Vitro and In Vivo

To test whether TGF- β executes its tumor promoting effects on MC38 cells, and LY2109761 can block these effects, we performed growth assay by cell counting and migration/invasion by in vitro Boyden chamber assays. Both exogenous and endogenous TGF- β induced proliferation (Figure 2A), migration (Figure 2B) and invasion of MC38 cells through either collagen membrane (Figure 2C) or matrigel barrier (Figure 2D). LY2109761 attenuated these endogenous or exogenous TGF- β -induced pro-oncogenic effects. Similarly, in wound healing assays, TGF- β accelerated wound closure in MC38 cells, whereas LY2109761 inhibited TGF- β -induced cell motility (Figure 2E). These results suggest that the TRKI, LY2109761 efficiently inhibits TGF- β -induced cell migration and invasion. In anchorage-independent growth assay, we observed that LY2109761 reduced exogenous and endogenous TGF- β -induced colony formation (in both number and size) by MC38 cells (Figure S2). We also observed that LY2109761 significantly reduced subcutaneous tumor growth when compared with control mice (Figure 2F). The inhibition of TGF- β signaling in vivo was confirmed by reduced phosphorylation of Smad2 in tumors of LY2109761-treated mice (data not shown). These results suggest an antitumor effect of LY2109761 in blocking both endogenous and exogenous TGF- β -mediated pro-oncogenic effects.

Blocking Endogenous TGF- β Signaling in MC38 Cells by LY2109761 Significantly Reduces Liver Metastasis

To examine whether MC38 cells metastasize to the liver, and LY2109761 can inhibit this metastasis, luciferase expressing-MC38 cells were injected into spleens of syngeneic C57BL/6 mice to generate liver metastasis. Mice receiving LY2109761 treatment showed significant reduction in the rate of metastatic foci formation as well as their growth and spread in the liver as determined by bioluminescence imaging, liver size and weight (Figure 3A & C). The integrated CT/microPET scan effectively showed the reduction in liver metastasis following treatment with LY2109761 (Figure 3B). LY2109761 reduced Smad2 phosphorylation in liver tumors in treated mice when compared with control mice, although levels of total Smad2 were similar (Figure 3D). This metastasis model was also used to determine the effect of LY2109761 on survival of mice. We observed that the mean survival of LY2109761-treated mice was

longer when compared with control mice (Figure 3E). These results indicate that blocking TGF- β signaling by TRKI suppresses colon cancer liver metastasis, and prolongs survival of mice bearing tumors.

LY2109761 Treatment Increases E-cadherin and Reduces MMP-9, nm23 and uPA Expression in Tumors

To gain insight into the molecular mechanism of how LY2109761 reduces liver metastasis of MC38 cells, we tested the expression of cell proliferation, adherens junction and migration/invasion related proteins in liver metastases. Although we observed higher E-cadherin expression in liver metastases of mice treated with LY2109761 (Figure 4A), β -catenin was slightly increased. We observed that LY2109761 reduced MMP-9 in liver metastases of treated mice, whereas the MMP-2 level remained unchanged (Figure 4A). The level of nm23 is increased in highly metastatic malignancies.^{16, 17, 18} We observed that LY2109761 significantly reduces nm23 expression in liver metastases of treated mice when compared with control mice (Figure 4A). We also observed that LY2109761 decreases the expression of uPA and COX-2 in liver metastases, which may contribute to the progression and metastasis of colorectal cancers (Figure 4A). The levels of cell-cycle regulatory proteins, Cdk2 and Cyclin D1 were reduced in liver metastases of LY2109761-treated mice (Figure 4B), whereas Cdk4 expression remained mostly unchanged. This might decrease the phosphorylation of Rb protein (Figure 4B) and as a result, cell proliferation in liver metastases might be inhibited.

As LY2109761 induced E-cadherin expression in liver tumors, we decided to investigate whether it can induce E-cadherin expression in MC38 cells in culture. Indeed, LY2109761 induced E-cadherin in MC38 cells in a time-dependent manner (Figure 4C). This suggests that endogenous TGF- β signaling reduces the expression of E-cadherin in liver metastases and treatment of the mice with LY2109761 restores E-cadherin expression.

Our H&E staining revealed poorly differentiated spindle cells in liver metastases of control mice when compared with treated mice (Figure 5). We did not observe significant difference in apoptotic tunnel positive cells. In agreement with the Figure 3D, we observed that phospho-Smad2 was predominantly localized in the nucleus in liver metastases of control mice, whereas LY2109761 significantly decreased nuclear phospho-Smad2 staining (Figure 5), suggesting the inhibition of endogenous TGF- β signaling by LY2109761 in liver metastases. We observed increased membranous localization of both E-cadherin and β -catenin in liver metastases of LY2109761-treated mice (Figure 5). These results suggest that blocking TGF- β signaling by LY2109761 may reduce liver metastasis through the regulation of junction and migration/invasion related proteins.

Stable Smad4 Expression in MC38 Cells Reverses TGF- β from Tumor Promoter to a Tumor Suppressor

To investigate the role of Smad4 re-expression on TGF- β signaling and tumorigenicity in MC38 cells, we generated stable Smad4 clones (Figure 6A). TGF- β induced functional complex formation between Smad2/3 and Smad4 in all three Smad4 stable clones (Figure 6B, top panel). TGF- β induced growth of MC38 and vector control clone as shown in Figure 2A, whereas Smad4 re-expression in all three clones restored TGF- β -induced growth inhibition (Figure 6C). These results suggest that loss of Smad4 in MC38 cells changes the role of TGF- β from growth inhibitor to a growth promoter. TGF- β induced migration (Figure 6D) and invasion (6E) of MC38 cells and vector control clone, whereas stable expression of Smad4 in the clones reduced basal invasive ability of MC38 cells and TGF- β treatment could not increase invasion of these Smad4 clones. Therefore, these data suggest an important role of Smad4 in controlling TGF- β -induced migration and invasion of MC38 cells.

Restoration of Smad4 in MC38 Cells Decreases Tumorigenicity and Metastasis to the Liver

To determine the effect of loss of Smad4 in colorectal cancer, we performed subcutaneous injection of these clones into C57Bl/6 mice. We observed that Smad4 clones showed reduced tumor growth when compared with the vector control clone (Figure 7A). We next tested the effect of Smad4 on metastatic ability of MC38 cells by splenic injection metastasis model. Interestingly, we observed significantly reduced liver metastases in mice injected with stable Smad4 clones when compared with that in mice injected with control cells (Figure 7B & C). These data indicate that loss of Smad4 in MC38 cells plays an important role in increasing tumorigenic and metastatic potential of these cells.

Smad4 inhibits tumorigenicity and metastasis of SW620 Human Colon Cancer Cells

In order to validate the effect of loss of Smad4 expression in human colon carcinoma, we generated stable Smad4 clones using SW620 cells (lacking Smad4 expression) (Figure 8A). We observed that all three stable Smad4 clones showed induced basal p3TP-Lux reporter activity as compared to SW620 and vector control clone, which did not increase further in response to TGF- β (Figure S3C). Re-expression of Smad4 in stable clones inhibits wound closing compared to controls (Figure 8B), and TGF- β had no effect on wound closing. In Boyden chamber assay, we observed that all three stable Smad4 clones showed reduced invasion through the collagen layer with or without TGF- β treatment compared to SW620 cells and vector control clone (Figure 8C). Taken together, these data suggest an important role of Smad4 in inhibiting the migration/invasion of SW620 cells.

In vitro soft agarose assay revealed that re-expression of Smad4 reduced the size and number of colonies by approximately two folds (Figure S3A & B). We also observed that all three stable Smad4 clones showed reduced tumor growth and size in nude mice compared to control cells injected mice (Figure 8D). In the splenic injection model of metastasis, we observed that 100% mice injected with SW620 cells and 80% mice injected with vector control cells produced liver metastasis (Figure 8E & F). Stable Smad4 clones injected mice showed significantly reduced liver metastasis. In addition, 100% mice injected with SW620 cells or vector control clone produced lymph node metastases, whereas only 20–40% mice injected with stable Smad4 clones produced much smaller lymph node metastases (Figure 8F). Interestingly, we observed that SW620 cells secrete good amount of endogenous TGF- β , whereas Smad4 expression reduces TGF- β secretion (Figure S3D). These results suggest that loss of Smad4 in SW620 cells is crucially involved in the metastasis of colorectal cancer and switches TGF- β from tumor suppressor to a tumor promoter.

Discussion

It is widely believed that Smad-dependent pathway is involved in TGF- β tumor suppressive functions, whereas activation of Smad-independent pathways coupled with the loss of tumor suppressor function of TGF- β is important for its pro-oncogenic effects. In the later stages of almost all epithelial cancers, high levels of TGF- β promote tumor growth, invasion and metastasis. The exact mechanism of this functional shift still remains elusive. Smad4 mutation and its downregulation in colorectal cancer are directly correlated to poor prognosis and increased metastasis.^{8,9} In contrast, the tumor suppressor Smad pathway has been shown to mediate the pro-metastatic function of TGF- β .^{11, 12, 13} Therefore, the role of Smad pathway in TGF- β -mediated metastasis remains poorly understood and controversial. Here we have demonstrated, for the first time we believe, that inactivation of Smad-dependent signaling through the loss of Smad4 in colorectal cancer is important for the functional switch of TGF- β from tumor suppressor to a promoter of tumorigenicity and metastasis. Interestingly, this study also provides molecular mechanism underlying the biological effects of the TRKI.

In addition to its effect on Smad pathway, TGF- β has been shown to activate the pro-oncogenic Ras/MAPK, PI3K/Akt, Rho kinase and JNK pathways with low levels of input signals in a Smad-independent manner.² We have observed that TGF- β can activate MEK/ERK pathway in MC38 and SW620 cells (Figure 1C). Importantly, ERK pathway is known to be activated in a variety of cancers and reported to play a role during colorectal tumor invasion and metastasis. It is possible that the TGF- β mediated induction of tumorigenicity and metastasis in MC38 and SW620 cells may be at least partially through the activation of ERK pathway. Unlike functional inactivation of T β RI/II that can potentially compromise both Smad-dependent and independent pathways, Smad4 inactivation selectively takes out only the tumor suppressive axis of the pathway, allowing TGF- β to preferentially over-activate the Smad-independent pathways in cooperation with other pro-oncogenic pathways including activated K-Ras.³ To study the tumor promoting effects of TGF- β and to evaluate the role of Smad4 on these effects, we have used two cell lines lacking Smad4 expression and with (SW620) and without (MC38) K-Ras activation. Expression of Smad4 in these cells restores Smad2/3 and Smad4 complex formation and TGF- β -induced transcriptional regulation (Figure 1B). Interestingly, Smad2 is phosphorylated in MC38 cells (lacking Smad4 expression) by exogenous TGF- β (Figure 1A), and endogenous TGF- β may induce its localization in the nucleus in liver metastases (Figure 5). This is in agreement with the fact that nuclear import of Smad2 sometimes does not require Smad4.¹⁹ At this point it is not clear whether phosphorylation and nuclear localization of Smad2 have any potential role in liver metastasis in the absence of Smad4. These results indicate that TGF- β signaling in MC38 cells is intact upstream and downstream of Smad4. Absence of any activating mutations in K-Ras rules out the possibility of cross-talk between TGF- β and activated Ras in this cell line.

The ability of TGF- β to induce cell proliferation, tumorigenicity, migration/invasion in MC38 cells (Figure 2) in absence of functional Smad-dependent pathway can only be explained by activation of Smad-independent pathways. These pro-oncogenic effects of TGF- β are inhibited by the TRKI, LY2109761. These results suggest that endogenous TGF- β plays a tumor promoter role in colorectal cancer in the absence of Smad4 expression, which is in agreement with the previous report suggesting that loss of Smad4 is associated with the occurrence of liver metastasis in colorectal cancer patients.⁹ Most importantly, we found that LY2109761 reduced tumorigenicity and liver metastasis of these cells, thus prolonging the survival of metastatic tumor bearing mice (Figure 3). These studies convincingly support LY2109761 as a potential therapeutic drug that can be used in combination with other pre-existing regimens for the treatment of advanced colorectal cancer. In an attempt to gain insight into the molecular mechanism underlying the antitumor effects of LY2109761, we have observed that LY2109761 induce the expression of E-cadherin (Figure 4A and 5), which is known to be critical for malignant progression of epithelial tumors.²⁰ We have observed that MC38 cells produce significant amount of TGF- β (Figure S3D) that activates endogenous TGF- β signaling in liver metastases as determined by Smad2 phosphorylation (Figure 3D) and its nuclear translocation (Figure 5). Endogenous TGF- β signaling in liver metastases seems to play a role in downregulating E-cadherin, the expression of which is restored by LY2109761. This is supported by the fact that LY2109761 treatment of MC38 cells induces E-cadherin expression (Figure 4C). Apart from this, LY2109761 also down-regulated pro-oncogenic/pro-metastatic proteins like MMP-9, uPA, and COX-2; all of which are known to be induced by TGF- β and in turn promote colorectal cancer progression. Although, reduced level of nm23 expression has been shown to be associated with aggressive tumor behavior²¹, high levels of nm23 expression were also noted in the advanced stages of colon, breast and thyroid carcinomas.^{16, 17, 18} In addition, in a related study nm23 has been shown to be upregulated in liver metastasis in a splenic injection model.¹⁵ Downregulation of nm23 by LY2109761 in vivo may contribute to its antitumor activity. In addition, levels of Cdk2 and Cyclin D1 were reduced in liver metastases of LY2109761-treated mice (Figure 4B), thus resulting in a decrease in pRb

phosphorylation. Therefore, regulation of these proteins by the TRKI, LY2109761 may be important for inhibiting cell proliferation, migration and invasion in liver metastasis.

The median overall survival of colorectal cancer patients with loss of Smad4 expression is 1.7 years, whereas for patients with high Smad4 levels, it is more than 9 years.¹⁰ When Smad4 was stably re-expressed in MC38 and SW620 cells, Smad-dependent pathway was adequately restored, which acted in a dominant manner to override the effects of Smad-independent pathways. Smad4 expression in these cell lines reversed the function of TGF- β from an inducer of proliferation and migration to an inhibitor. As MC38 cells are of murine origin, we used the SW620 human adenocarcinoma cells to validate our study and make it relevant to human disease. Indeed, Smad4 stable re-expression in SW620 cells inhibited cell migration/motility, reduced tumorigenicity in a tumor xenograft model, and also exhibited significantly reduced metastasis to the liver and the lymph nodes. However, only Smad4 expression in SW620 cells did not restore TGF- β -induced growth inhibition (data not shown). This is in agreement with the previous study suggesting the requirement of Smad4 and Ras phosphorylation-resistant Smad3 to restore TGF- β -mediated antiproliferative response in SW480 cells (with activated K-Ras and no Smad4 expression).²² This could be due to the fact that Ras activation is able to eliminate TGF- β antiproliferative responses but not other responses mediated by Smad4 that constrain colon tumor migration, invasion and metastasis. Our results are in agreement with our previous report suggesting that blockade of Smad pathway by overexpression of the inhibitory Smad, Smad7 in a colon adenocarcinoma cell line induces liver metastasis.¹⁵ TGF- β induces motility/migration of MC38 cells (Figure 2), whereas SW620 cells are unaffected by TGF- β (Figure 8), although the endogenous TGF- β level in MC38 cells is higher than that in SW620 cells (Figure S3D). It is possible that due to activated Ras signaling in SW620 cells (MC38 cells have wild type K-Ras) the basal motility is high, which is not regulated by TGF- β . Interestingly, we have observed that restoration of Smad4 expression in SW620 cells decreased the amount of secreted TGF- β (Figure S3D) that may play a role in attenuating the pro-oncogenic functions. Our data supports a causal connection between the loss of Smad4 expression in colorectal cancer and a more aggressive tumor phenotype.

In summary, although previous studies have suggested a pro-metastatic role of Smad signaling in breast cancer and melanoma metastasis to lung and bone, the present results indicate that loss of Smad4 expression in colorectal cancer enhances tumorigenicity and metastasis to the liver irrespective of K-Ras activation. Thus, the loss of Smad4 in colorectal cancer appears to switch TGF- β from tumor suppressor to a tumor promoter, and this group of patients could potentially benefit from TGF- β receptor kinase inhibitor therapy. This study also provides a mechanism for the antitumor effects of these inhibitors in colorectal cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

R01 CA95195 and CA113519, NCI SPORE grant in lung cancer (5P50CA90949, project# 4) and Veterans Affairs Merit Review Award (to P.K.D).

This study was supported by R01 CA95195 and CA113519, NCI SPORE grant in lung cancer (5P50CA90949, project# 4), and Veterans Affairs Merit Review Award (to P.K.D). We thank Dr. Brian Wadzinsky for helping us with the PP2A activity assay.

Abbreviations

CRC colorectal cancer

TGF- β	Transforming growth factor- β
TRKI	TGF- β receptor kinase inhibitor
MMP-9	Matrix metalloproteinase-9
uPA	Urokinase Plasminogen Activator
COX-2	Cyclooxygenase-2
T β RI	TGF- β type I receptor
T β RII	TGF- β type II receptor
VEGF	Vascular endothelial growth factor
ERK	Extracellular signal-regulated kinase
JNK	c-jun N-terminal kinase
EMT	Epithelial-to-mesenchymal transition
PAI-1	Plasminogen activator inhibitor-1
MAPK	Mitogen activated protein kinase
PI3K	Phosphatidylinositol-3 kinase

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Figure 1.

Smad-dependent signaling is inactivated in MC38 cells. (A) Lysates from TGF- β 1 (5 ng/ml) treated MC38 and FET cells (positive control) were subjected to immunoprecipitation (IP) for Smad2 and Smad3 followed by western blotting with anti-Smad4 antibody (top panel). Lysates were subjected to western blot analyses with antibodies against indicated proteins. (B) MC38 cells were co-transfected with CMV- β -gal and p3TP-Lux or (CAGA)₉ MLP-Luc with or without Smad4 expression construct. Cells were treated with TGF- β 1 (5 ng/ml) and/or LY2109761 (5 μ M). Luciferase activity was normalized to β -galactosidase activity and expressed as the mean $\pm$ S.D. of triplicate measurements. *P values <0.001 (For both reporters, the bars were compared as follows: third with first, fourth with third and sixth with fourth). (C) MC38 and SW620 cells were treated with TGF- β (5 ng/ml) for indicated time points, cell lysates were analyzed by western blotting with anti-ERK, phospho-ERK and -actin antibodies. Each experiment was repeated at least three times.

Figure 2.

LY2109761 suppresses TGF- β -induced pro-oncogenic activities. (A) MC38 cells were treated with TGF- β (5 ng/ml) with LY2109761 (5 μ M) for five days. Cells were counted every day and individual data points are presented as the mean $\pm$ S.D. of triplicate determinations. (B) MC38 cells that migrated through 8- μ m pores in transwell plates in 5 h were fixed, stained and presented as the mean $\pm$ S.D. of three wells. MC38 cells were allowed to pass through the collagen layer (C) and the matrigel barrier (D) in transwell plates. Data are presented similarly as above. For figures 2B, 2C and 2D, *P value < 0.01 (Treatment with TGF- β and LY together compared to treatment with TGF- β alone) and **P values < 0.001 (Treatment with LY compared to treatment with TGF- β) (E) Wound healing assay was performed with MC38 cells as described in Materials and Methods. Original magnification, 200X. (F) MC38 cells were injected subcutaneously into the right flanks of C57BL/6 mice. LY2109761 or vehicle was administered orally twice a day. Tumors were measured and plotted as the mean volume $\pm$ S.D..

Figure 3.

LY2109761 inhibits liver metastasis and prolongs survival. (A) Luciferase expressing-MC38 cells were injected into the spleens of C57BL/6 mice. Control mice (n=8) received vehicle and the treatment group was given (n=8) LY2109761 orally twice a day. Liver metastasis was monitored by bioluminescence imaging and images taken 23 days after splenic injection are presented. This experiment was repeated twice with similar results. (B) Integrated CT/microPET scan effectively shows reduction in liver metastasis following treatment with LY2109761. (C) Macroscopic liver metastases from control and LY2109761-treated mice are shown (top panel). LY2109761 reduced metastatic liver weight by more than 50% (bottom panel). *P values <0.001. (D) Lysates from livers of control and LY2109761-treated mice were analyzed with anti-phospho-Smad2 and anti-Smad2 antibodies. (E) Survival of mice treated with LY2109761 (n=6) or vehicle (n=6) was assayed after injecting MC38 cells into spleens of C57BL/6 mice. This experiment was repeated two times. *P<0.05

Figure 4. LY2109761 induces E-cadherin, and suppresses MMP-9, nm23, uPA and COX-2 and regulates cell cycle regulatory proteins. (A) & (B) Protein lysates from livers of control mice and LY2109761-treated mice were analyzed by western blotting as indicated. β -actin was used as loading control. NS: non-specific band. (C) Lysates from MC38 cells treated with LY2109761 (5 μ M) were analyzed by western blotting with anti-E-cadherin antibodies.

Figure 5.

Immunohistochemical analyses were performed using liver metastases of control and LY2109761-treated mice as described previously.¹⁵ Paraffin embedded blocks were prepared for serial sections and slides were processed for standard staining with H&E and Tunnel. Immunohistochemical analyses were performed with indicated antibodies. Original magnification, 630X.

Figure 6.

Smad4 expression in MC38 cells re-establishes TGF- β -induced growth inhibition, and reduces migration and invasion. (A) Three stable Smad4 clones in MC38 cells were analyzed for Smad4 expression. (B) Immunoprecipitation assay was performed with anti-Smad2/Smad3 antibodies using lysates from TGF- β -treated MC38 cells/clones, followed by western blotting with anti-Smad4 antibody (top panel). Cell lysates were also analyzed for expression of Smad proteins as indicated. (C) For cell growth assay, cells from above clones were treated with 5 ng/ml TGF- β for five days and counted. Cell numbers in presence of TGF- β are presented considering the respective untreated cells as 100%. Individual data points are the mean $\pm$ S.D. of triplicate counts on fifth day. *P < 0.01. Migration (D) and invasion (E) assays were performed with above MC38 stable Smad4 clones in presence or absence of TGF- β (5 ng/ml). Data points are the mean $\pm$ S.D. of migrated cells in three independent wells. For figures 6D and 6E, *P < 0.01 (All three Smad4 clones were compared with the parental and vector control MC38 cells both in presence or absence of TGF- β).

Figure 7.

Stable re-expression of Smad4 reduces tumorigenicity and metastasis of MC38 cells. (A) Subcutaneous tumors were generated by the clones and tumor volumes were measured. Growth curves for tumors are presented as the mean $\pm$ S.D. of five tumors. * $P < 0.03$, ** $P < 0.05$, *** $P < 0.01$ (B) Liver metastasis was examined 21 days after injecting MC38 cells/clones into spleens of C57BL/6 mice as described above. (C) The graph shows average of the ratio between metastatic liver weight and total body weight of mice in each group ($n=4$). * $P < 0.001$. This experiment was repeated two times.

F

Effect of Smad4 on inhibition of both liver and lymph node metastasis

Cells	No. of mice with liver metastasis	No. of foci (mean $\pm$ SE)	No. of mice with lymph node metastasis
SW620	7/7 (100%)	1.85 $\pm$ 1.12	7/7 (100%)
Vector	4/5 (80%)	1.20 $\pm$ 0.5	5/5 (100%)
Clone# 17	0/5 (0%)	0.00 $\pm$ 0.0	2/5 (40%)
Clone# 48	1/5 (20%)	0.20 $\pm$ 0.4	1/5 (20%)
Clone #53	1/5 (20%)	0.20 $\pm$ 0.48	2/5 (40%)

Figure 8.

Stable expression of Smad4 in SW620 cells reduces migration, invasion, tumorigenicity and metastasis. (A) Expression of Smad4 in stable clones generated from SW620 cells with CT26 cells as positive control. (B) Wound healing assay was performed using above clones as described in Materials and Methods. (C) Invasion assay using SW620 clones was done as described above. Data are presented as the mean $\pm$ S.D. from three independent wells. * $P < 0.001$ (Smad4 clones with TGF- β treatment compared to parental and vector control cells with TGF- β treatment), ** $P < 0.01$. (Smad4 clones without TGF- β treatment compared to parental and vector control cells without TGF- β treatment) (D) In vivo tumorigenicity of SW620 clonal cells was assessed. The growth curves for tumors are presented as the mean $\pm$ S.D. of five tumors. * $P < 0.05$, ** $P < 0.01$ (Smad4 clones compared with controls). (E) SW620 clonal cells were injected into spleens of athymic nude mice ($n=5$, for parental cells $n=7$). Eight weeks

after injection, mice were sacrificed and macroscopic liver metastasis or lymph node metastasis was assessed, as shown by circles in one mouse from each group. This experiment was repeated twice. (F) Data represent the effect of Smad4 re-expression on liver and lymph node metastasis. Number of mice with liver (including average number of foci per mouse) and lymph node metastases have been shown. A, $P < 0.001$, B, $P < 0.002$ compared to control by Student's t-test.