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Smad4-Mediated Signaling Inhibits Intestinal Neoplasia by Inhibiting Expression of β -Catenin

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Abstract

Background & Aims—Mutational inactivation of APC is an early event in colorectal cancer (CRC) progression that affects the stability and increases the activity of β -catenin, a mediator of Wnt signaling. CRC progression also involves inactivation of signaling via transforming growth

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factor (TGF) β and bone morphogenetic protein (BMP), which are tumor suppressors. However, the interactions between these pathways are not clear. We investigated the effects of loss of the transcription factor Smad4 loss on levels of β -catenin mRNA and Wnt signaling.

Methods—We used microarray analysis to associate levels of *Smad4* and β -catenin mRNA in colorectal tumor samples from 250 patients. We performed oligonucleotide-mediated knockdown of Smad4 in human embryonic kidney (HEK293T) and in HCT116 colon cancer cells and transgenically expressed Smad4 in SW480 colon cancer cells. We analyzed adenomas from (*APC* Δ 1638/+) and (*APC* Δ 1638/+) $\times$ (*K19Cre*^{ERT2}*Smad4*^{lox/lox}) mice using laser-capture microdissection.

Results—In human CRC samples, reduced levels of *Smad4* correlated with increased levels of β -catenin mRNA. In Smad4-depleted cell lines, levels of β -catenin mRNA and Wnt signaling increased. Inhibition of BMP or depletion of Smad4 in HEK293T cells increased binding of RNA polymerase II to the β -catenin gene. Expression of Smad4 in SW480 cells reduced Wnt signaling and levels of β -catenin mRNA. In mice with heterozygous disruption of *Apc* (*APC* Δ 1638/+), Smad4-deficient intestinal adenomas had increased levels of β -catenin mRNA and expression of Wnt target genes, compared with adenomas from *APC* Δ 1638/+ mice that expressed Smad4.

Conclusions—Transcription of β -catenin is inhibited by BMP signaling to Smad4. These findings provide important information about the interaction among TGF- β , BMP, and Wnt signaling pathways in CRC progression.

Keywords

Colon Cancer; Mouse Model; Intestinal Epithelium; signal transduction; carcinoma

Introduction

The central mediator of downstream Wnt signaling, β -catenin, is normally sequestered at the adherens junctions (E-cadherin, p120, β -catenin, α -catenin). Free cytoplasmic β -catenin is rapidly targeted for degradation through protein complexes that contains adenomatous polyposis coli (APC), Axin, and GSK3 β . However, in the presence of Wnt ligand, GSK3 β is inhibited resulting in accumulation of cytoplasmic β -catenin. This increase in cytoplasmic β -catenin results in nuclear translocation of β -catenin where it associates with LEF/TCF transcription factors to activate Wnt target genes¹.

Tight regulation of Wnt signaling within the normal intestinal tract is critical for maintenance of normal intestinal stem cell function and tissue homeostasis². Mutational inactivation of APC results in increased β -catenin protein levels with concomitant activation of the downstream Wnt signaling pathway and is an early event in colorectal cancer progression³. Inactivation of APC alone does not appear sufficient to fully activate Wnt signaling or to convey an invasive and metastatic behavior in tumor cells^{4,5}. It is likely that other mutational events or extrinsic factors may be necessary for maximal activation of the Wnt pathway to promote invasion and metastasis. Little is known about regulation of β -catenin levels outside of the paradigm of post-translational regulation. In particular, a role for regulation of β -catenin mRNA expression as an important mechanism to modulate Wnt signaling and colon cancer progression has not been described.

The TGF- β /BMP/Smad4 pathway is a developmentally crucial pathway that is also frequently mutated in colon cancer. BMP antagonists are expressed in the intestinal stem cell niche while BMP and TGF- β signaling activity increases as cells differentiate and migrate along the intestinal gland toward the intestinal lumen^{2,6–8}. Recent reports have included the BMP antagonist Noggin as a requisite factor within media for *in vitro* culture of isolated intestinal stem cells⁹. In colon cancer, TGF- β Receptor Type II (T β RII) is mutated

in >55% of cases¹⁰ and BMPRI/RII is mutated in >70% of cases¹¹ while Smad4 mutations are thought to occur late in 20–30% of cases^{12,13}. In addition, germline mutations in *Smad4* and *BMPRI1A* genes are frequently found in patients with Juvenile Polyposis Syndrome, a condition which predisposes patients to developing intestinal adenocarcinoma^{14,15}. Loss of Smad4 function in the presence of *Apc* mutation in mice markedly accelerates tumor progression¹⁶, but the mechanism of this cooperative interaction has not been fully defined.

Both β -catenin activation and Smad4 mutations occur frequently in colon cancer, yet the interaction between these signaling pathways in normal intestinal crypts and in colon cancer biology is unclear. In the present study, we find that decreased expression of Smad4 in human colon cancer is associated with increased expression of β -catenin mRNA. When Smad4 loss is induced in mouse intestinal tumor models, we observe increased expression of β -catenin mRNA and protein and associated increases in the mRNA expression of Wnt target genes, *c-Myc* and *Axin2*. In cultured colon cancer and human embryonic kidney (HEK293T) cells, both inhibition of Smad4 expression and inhibition of BMP receptor signaling cause similar increases in β -catenin mRNA expression and canonical Wnt signaling. The increase in β -catenin expression in response to Smad4 depletion or Noggin treatment is associated with increased engagement of RNA polymerase II to *ctnnb1* (the β -catenin gene). Thus, in addition to the important role of post-translational modification of β -catenin in canonical Wnt signaling in intestinal neoplasia, up-regulation of β -catenin mRNA expression plays a role in further amplifying the Wnt signal after inhibition of BMP signaling or loss of Smad4 expression.

Results

Inverse correlation of Smad4 and β -catenin expression levels in human colorectal cancer

While loss of Smad4 expression is associated with poor clinical outcomes in colon cancer patients¹⁷, its precise role in tumor progression has not been fully determined. To determine whether low Smad4 expression is associated with increased β -catenin expression in colon cancer, we analyzed Smad4 and β -catenin mRNA expression in a microarray dataset representing 250 colorectal cancer patient tumor samples (Stage 1: n=33; Stage 2: n=76; Stage 3: n=82; and Stage 4: n=59) and ten normal adjacent colorectal tissue specimens (Supplemental Table 1). We observed a significant down-regulation of Smad4 expression in both early and late stage colorectal tumors when compared with normal colon mucosa (Supplemental Figure 1A, $P<.0001$ for all stages compared to normal [n=10]) and significant up-regulation of β -catenin (Supplemental Figure 1B, $P<.002$ for all stages compared to normal). To examine if Smad4 and β -catenin mRNA expression levels are inversely correlated on a case by case basis, Pearson correlation tests were performed on the microarray data set. While there was no significant correlation when examining all 250 cases (Supplemental Figure 1C, $P<.09$), a significant inverse correlation was observed when examining Stage 1 and 2 cases (Supplemental Figure 1D, $P<.01$). These data suggest that with loss of Smad4 expression in colorectal cancer there is an increase in β -catenin mRNA expression levels.

Smad4 depletion in cultured epithelial cells results in increased β -catenin expression and activation of TOPFlash activity

Since the prevailing paradigm for regulation of β -catenin expression is post-translational, we were surprised to find that increased β -catenin mRNA is associated with Smad4 loss in human colorectal cancer samples. We utilized HCT116 colon cancer cells and Human Embryonic Kidney 293T (HEK293T) cells to determine whether loss of Smad4 expression results in increased expression of β -catenin mRNA and protein in epithelial cells. HCT116 cells are human colon cancer cells that are *Smad4*, and *APC* wild type^{18,19} but have

mutations in β -catenin (Ser45 del)¹⁸ and *TβRII*²⁰. HEK293T cells are immortalized human embryonic kidney epithelial cells that have intact Wnt and TGF- β /BMP family signaling pathways. When compared with scrambled siRNA treatment, Smad4 siRNA-treated HCT116 cells showed a modest increase in β -catenin protein levels (Figure 1A), along with a two-fold increase in β -catenin mRNA expression (Figure 1B), and a 6-fold increase in TOPFlash activity (Figure 1C). TOPFlash is a TCF reporter plasmid that measures activation of Wnt signaling²¹. In HEK293T cells, knock-down of Smad4 expression also caused a modest increase in β -catenin protein expression (Figure 1D), a 4-fold increase in β -catenin mRNA (Figure 1E), and a significant increase in TOPFlash activity (Figure 1F). Both cell lines showed increased TOPFlash activity with Wnt3a treatment, and both cell lines displayed a slight but not significant ($P < .15$) increase in TOPFlash activity when Smad4 siRNA treatment is combined with Wnt3a treatment. These data show that inhibition of Smad4 expression results in increased β -catenin mRNA along with increased activation of a Wnt/ β -catenin-activated reporter plasmid, providing functional evidence of nuclear β -catenin activity, in both HCT116 and HEK293T cell lines. The two-fold increase in TOPFlash activity in HEK293T cells as compared with the 6-fold increase in HCT116 cells is likely due to more rapid turnover of β -catenin in the absence of the stabilizing N-terminal mutation and an intact β -catenin destruction complex in the HEK293T cells.

Smad4 restoration suppresses β -catenin mRNA expression and represses TOPFlash activity in β -catenin dependent manner

The converse to blocking cellular Smad4 expression is restoration of *Smad4* to cells that lack Smad4 expression. SW480 cells have a deletion in one *Smad4* allele and splice site variant *Smad4* mutation in the other allele¹⁹. We previously reported that restoration of Smad4 into Smad4 deficient SW480 cells suppressed expression of the β -catenin/Wnt target gene, claudin-1, and that Smad4 expression also inhibited TOPFlash activity²². Tian et al.,²³ subsequently reproduced our findings and provided data suggesting that constitutive Smad4 expression in SW480 cells reduced β -catenin mRNA levels and decreased nuclear β -catenin immunoreactivity. When Smad4 is transiently transfected into SW480 cells (Figure 2A), we observed a significant ($P < .02$) decrease in β -catenin mRNA and protein levels (Figures 2A, B), and a dose-dependent repression of TOPFlash activity (Figure 2C). In contrast, Smad4 did not suppress TOPFlash activity in SW480 cells when co-transfected with β -catenin driven by a heterologous promoter (Figure 2D). Thus, these data support a model whereby restoration of the Smad4 signaling pathway inhibits β -catenin expression and that the decrease in β -catenin accounts for the decrease in TOPFlash activation.

Restoration of Smad4 and BMP signaling is associated with suppression of Wnt signaling

Prior work supports an important role for BMPs 1,2,5, and 7 in the modulation of Wnt signaling and promotion of cellular differentiation in the intestinal crypt⁸. In order to determine the ligand dependence of TOPFlash activity regulation in SW480 colon cancer cells, we co-transfected Smad4 with a well-characterized BMP-specific Smad reporter plasmid, BRE-Luc²⁴, and assessed BMP-mediated transcriptional activity associated with restoration of Smad4 expression. Previous studies have shown that SW480 and HCT116 cells express BMPRII receptors and are responsive to BMP2 and BMP7²⁵. We selected BMP2 for these experiments because the BMP2 gradient is increased toward the luminal surface of the colonic crypt². BRE-Luc activity is increased with Smad4 restoration (Figure 3A, Lane 5), and this activity was augmented with the addition of exogenous BMP2 ligand (Figure 3A, Lane6). Conversely, addition of exogenous Noggin, a pan-BMP antagonist, caused a decrease in steady state BRE-Luc activity in SW480 cells with Smad4 expression restored even in the presence of exogenous BMP2 ligand (Figure 3A, Lanes 7, 8). These data suggest that autocrine and paracrine BMP signaling are restored in SW480 cells upon Smad4 re-expression. We and others have found SW480 cells to be refractory to TGF- β

treatment^{13,22}, but remain BMP responsive²⁶. To determine whether both BMP- and TGF- β -activated signaling results in suppression of TOPFlash, we used BMP and TGF- β responsive HEK293T cells (Figure 3B). BMP2 treatment of HEK293T cells suppressed TOPFlash activity but TGF- β treatment did not (Figure 3C). In HEK293T cells, we observed a modest, but significant, decrease in β -catenin mRNA levels after treatment with BMP2 and a modest, but significant, increase in β -catenin mRNA levels after Noggin treatment (Figure 3D). Taken together, these experiments support the conclusion that Smad4 restoration/expression enables canonical BMP signaling to decrease β -catenin expression and inhibits Wnt signaling.

BMP signaling regulates RNA polymerase II activity of *ctnnb1*

To determine whether Smad4 regulates transcription of the *β -catenin* gene, we assessed RNA polymerase II bound to the 2nd exon (+460 to +579) by chromatin immunoprecipitation (ChIP) in HEK293T cells. We observed a significant decrease in RNA polymerase II (RNA Pol II) binding at exon 2 of the *ctnnb1* gene in HEK293T cells 12 hours after treatment with BMP2 as compared with untreated cells. Conversely, treatment with the BMP antagonist, Noggin, caused an increase in RNA Pol II engagement with exon 2 of *ctnnb1* (Figure 4A). To examine if this release of transcriptional repression occurs through Smad4-dependent signaling, we depleted Smad4 using siRNA. We observed a significant increase in RNA polymerase II (RNA Pol II) pulled down at exon 2 of the *ctnnb1* gene upon Smad4 depletion; however, this increase was not augmented with combination of Smad4 depletion and Noggin treatment (Figure 4B). These data support the conclusion that transcription of *ctnnb1* is repressed by BMP signaling through Smad4 and that release of transcriptional repression occurs similarly when BMP signaling is inhibited at the receptor level by Noggin or by depletion of Smad4.

Smad4 loss promotes carcinogenesis in the presence of mutated tumor suppressor *in vivo*

In order to further examine the biological significance of the above observations, we employed genetically defined conditional mouse models of Smad4 depletion. For these experiments, we crossed Smad4^{lox/lox} mice²⁷ with K19Cre^{ERT2} mice²⁸, to generate K19Cre^{ERT2}Smad4^{lox/lox}. These mice can be induced with tamoxifen-treatment to undergo recombination of the *Smad4* gene with resultant loss of Smad4 expression, specifically in the stem cell compartments of small intestine, colon, pancreas, and liver. We observed that tissue-specific, conditional loss of Smad4 in colonic epithelium results in expansion of the zone of proliferative cells within the colonic crypt but not in the formation of neoplastic lesions (Supplemental Figure 2A–C). In response to Smad4 depletion induced by tamoxifen treatment in the APC ^{Δ 1638/+} background, mouse colonoscopy readily detected tumors in the colons of tamoxifen-treated mice by one month after treatment while the colons of vehicle-treated mice appeared entirely normal (data not shown). Smad4 depletion resulted in a ten-fold increase in the tumor burden in both the large and small intestine tumor burden by five weeks after tamoxifen treatment of (K19Cre^{ERT2}Smad4^{lox/lox}) $\times$ (APC ^{Δ 1638/+}) mice as compared to vehicle-treated control mice with intact Smad4 expression (Figure 5A, n=4 for vehicle and tamoxifen groups). IHC analysis of the polyps from tamoxifen-treated mice showed that all observed adenomatous lesions (n=12) were depleted of Smad4 protein expression and exhibited abundant nuclear β -catenin staining (Figure 5B, C), whereas adenomas from vehicle-treated mice retained Smad4 (Supplemental Figure 2D, E). Since (APC ^{Δ 1638/+}) mice rarely develop colonic adenomas²⁹, we performed β -catenin immunostaining on the small intestinal polyps from both vehicle-treated and tamoxifen-treated (K19Cre^{ERT2}Smad4^{lox/lox}) $\times$ (APC ^{Δ 1638/+}) mice. There was a significant (P<.01) increase in the percentage of epithelial cells that displayed nuclear localization of β -catenin by IHC in the tamoxifen-treated mice (Figure 5D and Supplemental Figure 3, black arrows

identify cells with nuclear β -catenin immunoreactivity, white arrows identify cells lacking nuclear β -catenin immunoreactivity).

Smad4 loss is associated with increased β -catenin mRNA levels and increased Wnt target gene expression in murine adenomas

qPCR analysis of mRNA from microdissected small intestinal adenomas from vehicle-treated and tamoxifen-treated mice demonstrated a 6-fold increase in β -catenin mRNA levels in adenomas depleted of Smad4 (Figure 5E) when compared with normal adjacent tissue. For adenomatous lesions in vehicle-treated mice, in which Smad4 was retained, there was only a 2-fold increase when compared to normal adjacent tissue (Figure 5F). This represents a significant ($P<.05$) increase in β -catenin mRNA levels when comparing lesions that have loss of Smad4 expression to those that have retained Smad4 expression. We also observed increased c-Myc (Figure 5E, $P<.05$) and Axin2 (Figure 5F, $P<.05$) mRNA expression in lesions that have loss of Smad4 expression when compared to those that retained Smad4 expression. These data indicate that Smad4 loss is associated with increased β -catenin mRNA and Wnt target gene expression levels in murine adenomas.

Murine model parallels β -catenin expression pattern observed in human colorectal cancer

To extend these observations, we examined Smad4 and β -catenin protein expression patterns in tumor tissue serial sections by immunohistochemistry (IHC) ($n=24$, demographics provided in Supplemental Table 2). Immunostaining for Smad4 and β -catenin was scored by grading intensity as 0-negative, 1-weak, 2-moderate, and 3-strong. Among these samples, 19 (~79%) patients retained Smad4 expression (Immunostaining score [IS]=1–3) while 5 (~21%) exhibited complete tumor cell loss of Smad4 expression (IS = 0). Among those samples exhibiting complete tumor cell Smad4 loss (IS=0), 4 (80%) had higher than median β -catenin IHC staining (IS>2) when compared with the other tumor samples. For those tumor samples retaining Smad4 expression, only 5 (~25%) samples had higher than median β -catenin immunostaining (Supplemental Table 3). Supplemental Figure 4A shows a representative colon tumor section showing predominant Smad4 nuclear staining within the glandular carcinoma cells (black arrows). The serial section (Supplemental Figure 4B) is stained for β -catenin and shows that cells with predominant nuclear staining of Smad4 (black arrows) are associated with membrane-localized β -catenin staining. In contrast, a representative section from a colon tumor lacking Smad4 expression (Figure 1E) shows Smad4 staining exclusively stromal cells (Supplemental Figure 4C) and strong nuclear and cytoplasmic β -catenin staining in the serial section (Supplemental Figure 4D). Similarly, in the tumor retaining Smad4 expression, cells lacking nuclear Smad4 staining (Supplemental Figure 4A, white arrows) correspond with strong nuclear and cytoplasmic β -catenin staining (Supplemental Figure 4B, white arrows).

Discussion

The prevailing paradigm for regulation of β -catenin levels and Wnt signaling activity has been through the ubiquitin-proteasome post-translational regulation of β -catenin protein stability. In this report, we provide experimental evidence for BMP/Smad4-mediated repression of β -catenin mRNA expression as an alternative mechanism for modulating the Wnt signal. We have shown that loss of Smad4 expression results in increased β -catenin mRNA expression and amplification of Wnt signaling both *in vitro* and *in vivo*. While other studies have provided evidence for regulatory cross-talk between TGF- β family and Wnt signaling pathways, the mechanisms by which this occurs have not been fully determined^{22,23}. A recent example of this cross-talk implicates up-regulation of a novel Wnt target gene, BAMBI, in the inhibition of TGF β signaling and poor prognosis in colorectal cancer patients³⁰. Increased *BAMBI* expression through Wnt signaling is consistent with the

concept of a positive feedback loop in the context of colorectal cancer that would suppress TGF- β pathway signaling to further amplify Wnt pathway signaling and augment tumor progression.

In our human colon cancer studies, we observe complete loss of Smad4 expression in ~25% of samples in agreement with others^{12,13}. Our data extend the prior observations to suggest that reduced Smad4 expression correlates with increased expression of both β -catenin mRNA and protein in colorectal cancers. Several prior studies have reported that decreased Smad4 immunoreactivity in colorectal cancer is associated with worse prognosis^{17,31,32}. In fact, based on semiquantitative IHC scoring, Stage III colorectal cancer patients whose tumors exhibited low Smad4 levels had a median overall survival of only 1.7 years, whereas patients with high Smad4 tumor levels had a >9 year median survival¹⁷. Our observations that loss of Smad4 has a profound effect on the multiplicity and progression of intestinal adenomas in genetically modified mice raises the question of whether disruption of TGF- β /BMP signaling has a role in the progression of early human intestinal polyps. Mouse models of Juvenile polyposis with BMPRI1A deficient⁶ or Noggin overexpression³³ exhibit increased and nuclear localized β -catenin and amplified Wnt signaling in the intestinal polyps. The Vogelstein model places loss of heterozygosity at 18q and loss of Smad4 in the late adenoma phase of tumor progression³. While loss of Smad4 expression has not been demonstrated in early intestinal adenomas associated with *Apc* mutation, Hardwick et al.³⁴, demonstrated decreased BMP2 expression in early adenomas, which could result in a similar increase in β -catenin. It is likely that loss of Smad4 in more advanced adenomas may further amplify Wnt signaling and drive tumor progression.

Our findings support the conclusion that Smad4 exerts its effect on β -catenin expression at the level of β -catenin mRNA, with important downstream consequences in Wnt signaling. However, this effect of BMP/Smad4 signaling on β -catenin mRNA expression is not mutually exclusive with the well-described mechanism of post-translational regulation of β -catenin by APC, Axin2 and GSK3 β . For example, He, et al.⁶, implicated BMP mediated inhibition of Wnt activity through activation of PTEN, with inhibition of PI3Kinase and Akt activity. Blockade of BMP signaling resulted in inactivation of PTEN, activation of Akt with resultant inactivation of GSK3 β and accumulation of β -catenin⁶. Kosinski et al.², reported that the BMP inhibitor, Gremlin1, activates Wnt/ β -catenin signaling in normal intestinal epithelial cells (IEC-18). While intriguing, the non-canonical pathways implicated by He⁶ and Kosinski² do not explain the significant increases in β -catenin mRNA and protein expression that we observe when Smad4 expression is lost. Interestingly, targeted disruption of *Smad4* expression in the mouse hair follicle was recently shown to increase active cellular β -catenin and expression of the Wnt target³⁵, *c-Myc*, without increasing Akt activity or GSK-3 β phosphorylation. This is consistent with our observations but is in contrast to the observations by He, et al.⁶. Our findings are also consistent with recent work showing that conditional Smad4 loss within mouse dental mesenchymal cells results in increased β -catenin mRNA levels along with increased activation of canonical Wnt signaling³⁶. Thus, mounting evidence in multiple experimental systems supports the conclusion that Smad4-dependent regulation of *β -catenin* mRNA expression is a biologically significant mechanism.

In the mouse, the present study supports the conclusion that induced Smad4 loss in the intestinal epithelium stimulates the progression of intestinal neoplasia. Our mouse tumorigenesis results are consistent with the two-hit hypothesis for carcinogenesis as previously described by Knudson³⁷. Evidence put forth by Takaku, et al.¹⁶, demonstrated that combined mutations in *Apc* and *Smad4* accelerated tumor progression in comparison to mutation in *Apc* alone. Notably, despite mosaic recombination and loss of Smad4 within only 20–30% of the intestinal glands in our studies, we observed loss of Smad4 expression

in 100% of colonic and small intestinal adenomas examined in tamoxifen treated APC^{Δ1638/+}K19Cre^{ERT2}Smad4^{lox/lox} mice. While in vehicle treated isogenic mice, 100% of the adenomas, almost all of which are located in the small intestine, retained Smad4 expression. Importantly, we also observed a significant increase in expression of β -catenin mRNA, nuclear localization of β -catenin protein and increased expression of Wnt target genes *Axin2* and *c-Myc*³⁸ in Smad4 depleted lesions. Superinduction of *c-Myc* expression is particularly noteworthy in that expression can be transcriptionally activated by β -catenin/TCF but transcriptionally repressed by TGF- β /Smad signaling^{39,40}.

Our experimental results show that inhibition of BMP signaling or loss of Smad4 can similarly augment β -catenin levels through a transcriptional mechanism, thereby increasing Wnt signaling. Consensus binding elements for NF κ B, EGR1, SP1 and AP1 in the proximal β -catenin promoter have been reported⁴¹, and we have noted 12 potential consensus Smad binding elements at position within the 3500 bases 5-prime of the transcriptional start site of the *ctnnb1* gene. It will be of interest to determine whether Smad4 interacts with the β -catenin promoter to modulate its transcription.

In conclusion, we have shown that the impact of increased β -catenin mRNA associated with Smad4 loss is biologically significant, especially in the setting of *Apc* mutation, where the augmented Wnt signaling is associated with increased Wnt/ β -catenin target gene expression and an increased tumor burden. These findings provide potential insight into the important relationship between Smad4 loss in colon cancer and poor patient prognosis and late-stage disease^{17,31}. Further investigation into the precise mechanisms underlying our observations may provide new avenues to identify therapeutic targets.

Materials and Methods

Cell culture, transfection, and FACS Sort

SW480, HCT116, and Human Embryonic Kidney (HEK) 293T (HEK293-T) cells were obtained from American Type Culture Collection (see Supplementary Methods).

Fluorescence activated cell sorting (FACS) was performed on SW480 cells transiently co-transfected with pRK-5 Smad4 (0.8 μ g/mL) and pEGFP (1.0 μ g/mL). Cells were sorted using the FACS Aria System (BD Biosciences, San Jose, CA) gated at 488nm, 48hours post-transfection. The top 30% of GFP expressing cells were selected for RNA and protein isolation.

Immunoblots

See Supplementary Methods

RNA Isolation, Preparation, and Analysis

Qiagen RNeasy[®] kit (Valencia, CA) was utilized for RNA collection. All quantitative PCR reactions were done in quadruplicate and normalized to mock transfected samples for qPCR on HEK293T or HCT116 cells. Statistical analysis of fluorescence was performed using LightCycler 480 SW Version 1.5 (Roche, Indianapolis, IN). (see Supplementary Methods).

Chromatin immunoprecipitation (ChIP) Assay

ChIP assays were performed using the EZ-Magna ChIP (Millipore Cat. #17-408), according to manufacturer instructions. Isolated DNA was subjected to quantitative PCR with a SYBR Green PCR Kit (Applied Biosystems, Carlsbad, CA) using primers for β -catenin Exon 2. (see Supplementary Methods).

Mouse models

See Supplementary Methods

Laser Capture Microdissection

Small intestinal polyps from APC^{Δ1638/+}K19Cre^{ERT2}Smad4^{lox/lox} mice treated with vehicle (n=4) or tamoxifen (n=4) were frozen in OCT. Frozen sections (5μm) sections were cut and dehydrated prior to tissue isolation using the VeritasTM704 laser capture microdissection device (Arcturus Engineering, Mountain View, CA). RNA was then isolated from both polyp tissue areas as well as normal adjacent tissue areas using Qiagen Micro RNeasy RNA isolation kit (Valencia, CA). Isolated RNA with RIN values >6.5 was then amplified using WT-OvationTM Pico RNA Amplification System (NuGEN, San Carlos, CA) prior to qPCR analysis using the LightCycler 480 (Roche, Indianapolis, IN) according to manufacturer instructions. Expression of target genes in Smad4-expressing and Smad4 deficient polyps was normalized using gene expression in paired normal adjacent tissue.

Immunohistochemistry

See Supplementary Methods

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

IHC	Immunohistochemistry
HEK293T	Human Embryonic Kidney cells
APC	Adenomatous Polyposis
BMP	Bone Morphogenetic Protein
TGF-β	Transforming Growth Factor -β
R-Smads	Receptor associated Smads

K19

Cytokeratin 19

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Figure 1. Smad4 depletion in cultured epithelial cells results in increased β -catenin expression and activation of TOPFlash activity

Oligonucleotide (RNAi) mediated Smad4 knockdown conducted in (A–C) HCT116 and (D–F) HEK293T human colon cancer cells. (A, D) Results of western blots showing relative expression of Smad4, Smad2, β -catenin and β -actin 24 hours post-transfection with scrambled or Smad4-specific RNAi (B, E) qPCR results showing relative expression of β -catenin mRNA expression 24hours post-transfection with scrambled and Smad4-specific RNAi. The graph shows quantified β -catenin mRNA relative to levels in mock-transfected cells normalized to Pmm1 expression. TriPLICATE for each condition was performed with mean $\pm$ SEM displayed. Student's t-tests were performed to determine significance. (C, F) TOPFlash activity 24 hours post-transfection with scrambled or Smad4 specific RNAi, with and without treatment with Wnt3a conditioned media. The graph shows light units from induced TOPFlash activity normalized to constitutive Renilla activity, relative to mock-transfected cells. Significance determined by ANOVA.

Figure 2. BMP signaling suppresses TOPFlash activity and regulates β-catenin mRNA expression levels

(A) SW480 cells were transiently co-transfected with either BRE-Luc and pCMV-Script or pCMV-Smad4 and then treated with either vehicle 20ng/ml BMP2 and/or 200ng/ml Noggin for 24 hours. Relative luciferase activity is graphed. Significance was determined by ANOVA tests. (B) Western blot of parallel experiments of HEK293T cells. Protein lysate was taken 30 min and 24 hours after the indicated treatment to capture the early phosphorylation of R-Smads (p-Smad1, Smad1, p-Smad2, and Smad2) and the downstream effects of activation of the pathways (β-catenin and Id2). Representative β-actin taken at 24 hours is displayed. (C) HEK293T cells were treated with either vehicle, 200ng/ml BMP2, 5ng/ml TGF-β, or Wnt3a conditioned medium (1:1) and assayed for TOPflash activity at 24 hours post-transfection and 12 hours post-treatment. (D) Results of qPCR assays showing relative expression of β-catenin mRNA expression in HEK293T at 12 hours post-treatment with 20ng/ml BMP2, 200ng/ml Noggin or Wnt3a conditioned media. The graph shows quantified β-catenin mRNA relative to untreated cells. Duplicate for each condition was performed with mean $\pm$ SEM displayed for all. Significance determined by student's t-tests (C&D).

Figure 3. Smad4 restoration suppresses β -catenin mRNA expression and represses TOPFlash activity in a β -catenin dependent manner

((A) Representative results from three biological replicates of FACS sorted SW480 cells co-transfected with pRK-5Smad4 (0.8 μ g/mL) and pEGFP (1.0 μ g/mL). Protein isolated from GFP- (Smad4-null) and GFP+ (Smad4+) was run on western blot. (B) qPCR results from Smad4-null and Smad4+ cells from (A) showing relative β -catenin expression that was normalized to Pmm1 expression. (C) TOPflash activity 48hrs post-transfection with p-RK5 empty vector or p-RK5-Smad4 vector as indicated. Triplicate for each condition were performed. (D) TOPFlash reporter activity in SW480 cells and in SW480 cells transiently co-transfected with 0.2 μ g wild-type human β -catenin and 0.8 μ g p-RK5 empty vector or 0.8 μ g p-RK5-Smad4 (as indicated). Two biological replicates are displayed. Significance determined using student's t-test. Mean $\pm$ SEM are displayed with FOP control (C&D).

Figure 4. BMP signaling regulates RNA polII activity of *cttnb1*

HEK293T cells treated for 12 hours with BMP2, Noggin, or Wnt3a, as labeled, then nuclear lysates were prepared as described followed by immunoprecipitation with anti-RNA pol II antibody. **(A)** qPCR analysis of bound exon 2 of *cttnb1* using the LightCycler 480 System for analysis. **(B)** HEK293T cells transfected with either scrambled siRNA or Smad4 siRNA and after 24 hours were treated for 12 hours, as labeled. Nuclear lysates were prepared as described above, followed by immunoprecipitation with anti-RNA pol II antibody and amplification of exon 2 of *cttnb1* for qPCR analysis. Results presented are from two biological replicates run in triplicate.

Figure 5. Loss of Smad4 *in vivo* leads to increased tumor burden, levels of β-catenin mRNA, and downstream targets of Wnt Signaling

(A) Quantification of polyp burden observed on gross dissection in APC^{Δ1638/+}K19Cre^{ERT2} Smad4^{lox/lox} mice five weeks post-treatment (n=8 for both treatments). Serial sections (B, C) of APC^{Δ1638/+}K19Cre^{ERT2} Smad4^{lox/lox} polyp resected 5 weeks post-tamoxifen treatment. Photomicrographs were taken at 50x magnification. Scale bars equal 200μm (B) Smad4 immunostaining with outlined areas indicate corresponding Smad4-positive region. (C) β-catenin immunostaining with black arrows indicating areas of nuclear β-catenin staining. (D) Quantification of cells with positive nuclear β-catenin staining (defined as nuclear staining > cytoplasmic staining). Each data point represents the average percentage of positive cells within three high power fields for a single polyp (n=7 control polyps, n=7 tamoxifen polyps). Relative expression of (E) Smad4, c-Myc (F) β-catenin, Axin2 mRNA in vehicle (n=4) or tamoxifen (n=4) treated small bowel adenomas from APC^{Δ1638/+}K19Cre^{ERT2}Smad4^{lox/lox} mice. Expression of mRNA is displayed as fold changes (2^{ΔCt}) normalizing by the expression of target genes in normal adjacent tissue. Smad4 and c-Myc data are log transformed to display differences in a clear manner due to magnitude of difference. Significance determined by student's t-tests.