UNDERSTANDING THE ROLE OF SMAD4 IN INTESTINAL HOMEOSTASIS AND TUMORIGENESIS

By

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DEDICATION

These words of gratitude are penned for the individuals in my life who granted me the wisdom to persevere, no matter the obstacle, and without whose support none of this would have been possible.

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ABBREVIATIONS

β-cat	β-catenin
APC	adenomatous polyposis coli
Atoh1	atonal homologue 1
BMP	bone morphogenetic protein
CK1α	casein kinase 1α
CRC	colorectal cancer
DSS	Dextran Sodium Sulfate
E-cad	E-cadherin
EGF	epidermal growth factor
GALT	gut-associated lymphoid tissue
GDF	growth and differentiation factor
GEO	gene expression omnibus
Gli	glioma
GSK3β or GSK3	glycogen synthase kinase3 β
IHC	immunohistochemistry
IHH	Indian hedgehog

M cells	microfold cells
MIS	Muellerian inhibitor substance
mRNA	messenger RNA
NICD	notch intracellular domain
PAS	periodic acid schiff
qPCR	quantitative polymerase chain reaction
R-Smads	receptor smads
RANKL	receptor activator of nuclear factor kappa-B ligand
SHH	sonic hedgehog
siRNA	small interfering RNA
TCF/LEF	T cell factor/ lymphoid enhancer binder 1
TGF-β	transforming growth factor β
TGFα	transforming growth factor α

CHAPTER I

INTRODUCTION TO SIGNALING PATHWAYS IMPLICATED IN INTESTINAL HOMEOSTASIS AND INTESTINAL TUMOR DEVELOPMENT

Intestinal Epithelium Biology

The intestinal tract, simply put, is a muscular tube lined by a single layer of epithelium which is highly dynamic and has multiple functions. Within the digestive tract, the intestinal epithelium has unique absorptive functions as well as important barrier functions, forming the interface between the host and external environment. In order to simultaneously accomplish both absorptive and barrier functions, the intestine maintains a simple columnar epithelium with specialized apical and baso-lateral cellular structures for each task. Intestinal crypt/villus architecture is maintained in a dynamic homeostasis sustained by stem cells residing in the crypt which produce daughter cells that migrate toward the luminal aspect of the tract before being shed into the lumen after undergoing apoptosis/anoikis (Grossmann, Walther et al. 2002) (Figure 1.1A,B). By this process, most of the differentiated intestinal epithelium is replaced every 5-7 days from the stem cell compartment (Creamer, Shorter et al. 1961). While transiting the crypt/villus axis toward the intestinal lumen, cells pass through a transient proliferative compartment before differentiating into specialized enterocytes (absorptive), goblet, tuft, Paneth, enteroendocrine, microfold, and cup cells (Figure 1.1 A,B; tuft, microfold, and cup cells not depicted). A delicate balance must be maintained between the proliferative aspect of the intestinal crypt to replace cells being shed into the lumen and the differentiation aspect of the crypt which permits cells to perform their proper function in order to maintain homeostasis.

Reflecting function, the architecture of the intestinal tract varies significantly. The

proximal aspect of the small intestine (duodenum) is highly specialized for absorptive function reflected in the prominence of villi and microvilli structures that increase the

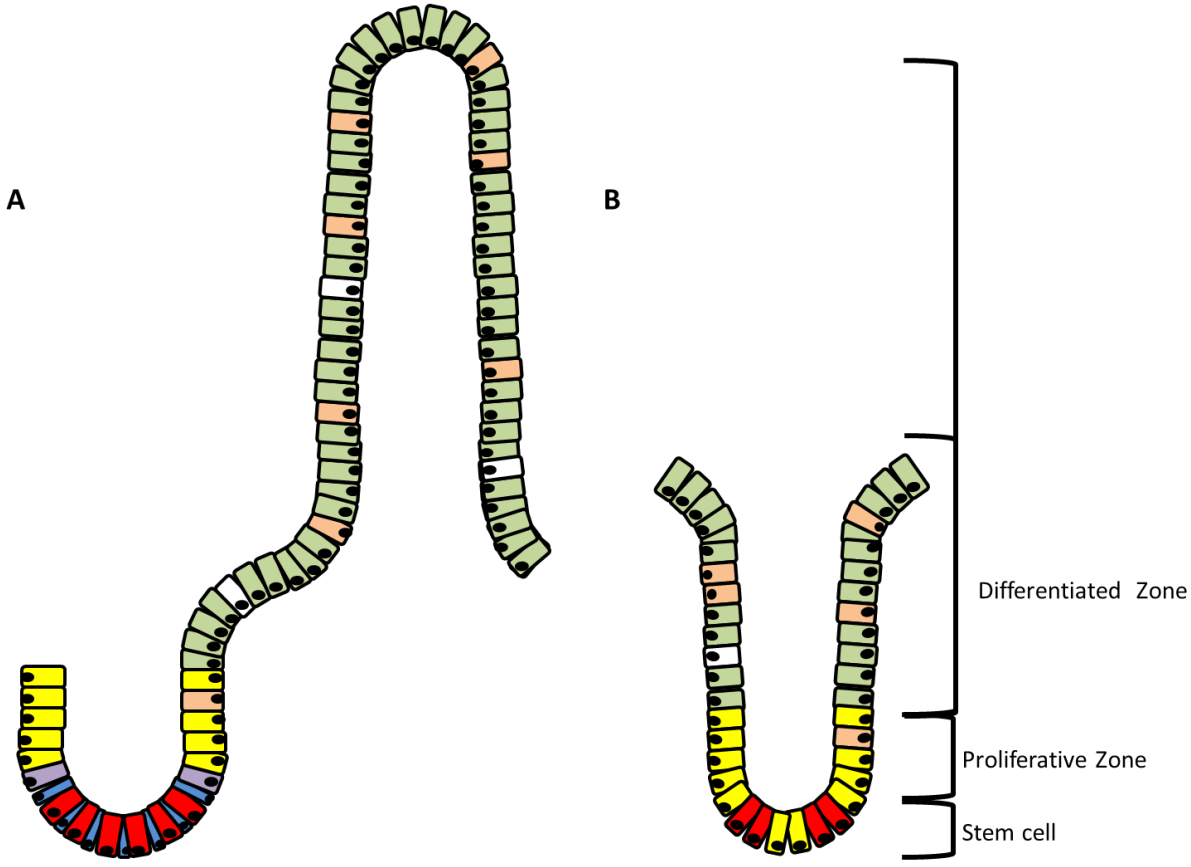


Figure 1.1. *Diagram of intestinal epithelium*. Depiction of epithelium in the small (A) and large (B) intestine showing a simple columnar epithelium. Stem cells are represented by red cells while Paneth cells within the small intestine (A) are represented by blue cells. 4+ stem cells are represented by purple cells in the small intestine (A). Secretory goblet cells and enteroendocrine cells are represented by orange and white cells, respectively. Green cells represent absorptive enterocytes. Yellow cells represent cells within the proliferative zone. Diagrams are not to scale.

absorptive surface area. Toward the more distal aspect of the small intestine (ileum), where absorptive need is reduced, villus structures are shorter and surface area is reduced. The architecture of the large intestine, which functions to re-absorb water, concentrate fecal material and lubricate feces, to prevent mechanical damage, lacks villus projections altogether (Figure 1.1A,B).

The cellular architecture of the intestinal crypt/villus is governed by specific signals triggered by positional cues arising from adjacent epithelial cells and from the adjacent tissue stroma. The central signaling pathway in determining cell fate decisions in the crypt is Notch signaling (discussed in greater detail below). Activation of Notch signaling within the intestinal tract directs a cell to differentiate into the absorptive enterocytes over the secretory lineage (Fre, Huyghe et al. 2005, Gerbe, van Es et al. 2011); whereas, inhibition of Notch signaling leads to secretory cell hyperplasia within the intestinal crypt (van Es, van Gijn et al. 2005). Enterocytes are the main absorptive cells; they function to absorb nutrients (ions, vitamins, amino acids, carbohydrates, and lipids) from the intestinal lumen, to secrete immunoglobulins, and to act as a barrier, and they comprise the majority of epithelial cells within the intestinal crypt. Goblet cells are found throughout the small and large intestine and function in secreting mucins that create a mucinous barrier within the intestinal tract to protect epithelial from microbial insult and mechanical stress (Specian and Oliver 1991). Goblet cells can be identified by expression of mucins (Muc2, etc.), Periodic Acid-Schiff (PAS) or Alcian blue stains (Takeyama, Dabbagh et al. 1999). Enteroendocrine cells are identified by expression of chromagranin A and secrete hormones and signaling molecules such as gastrin, ghrelin, somatostatin, cholecystokinin, serotonin, glucose-dependent insulinotropic peptide, glucagon-like peptides, and peptide YY (Moran, Leslie et al. 2008). Paneth cells are found strictly in the small intestine and appendix at the base of the crypt-villus axis, are marked by CD24, and unlike the other differentiated cells of the epithelium, remain in

the crypt for approximately three weeks (Garabedian, Roberts et al. 1997, Sato, van Es et al. 2011). Paneth cells function in the maintenance of stem cell niche as well as protecting the crypt base from microbial insult by secretion of anti-microbial peptides (such as crytidin-1 and lysozyme) (Ayabe, Satchell et al. 2000, Porter, Bevins et al. 2002). Tuft cells comprise relatively few of the epithelial cells within the intestinal tract (~0.5%) and their precise function is still not known (Gerbe, Legraverend et al. 2012). They can be identified by their expression of cytokeratin 18 and DCLK1 though the latter marker also marks a subset of enteroendocrine cells and quiescent stem cells (Hofer and Drenckhahn 1996, Gerbe, van Es et al. 2011). Microfold cells (M cells) are specialized epithelium that with enterocytes overlay gut-associated lymphoid tissues (GALTs), and permit transcytosis of antigens and microorganisms and present them to the underlying lymphoid tissue (Nicoletti 2000). M cells are stimulated by RANKL and identified by their expression of UEA-1, annexin V, SpiB, and GP2 expression and also express vimentin (de Lau, Kujala et al. 2012). Cup cells are limited mostly to the ileum of the small intestine and are characterized by shorter brush border, markedly weaker alkaline phosphatase expression compared to enterocytes and express vimentin like M cells though they do not present antigens to lymphoid tissue (Madara 1982, Fujimura and Iida 2001, Gerbe, Legraverend et al. 2012).

Intestinal Stem Cells

Within the intestinal epithelium, stem cells constantly undergo asymmetrical division to repopulate the differentiated epithelium while simultaneously maintaining the stem cell niche. Within the small intestine, there are data to support the existence of two separate stem cell populations: the columnar base cells wedged between Paneth cells that undergo steady division within the crypt and the +4 position stem cells that remain quiescent until a stimulus, such as injury, occurs signaling entry into the cell cycle

(Barker, van de Wetering et al. 2008). The stem cell niche within the large intestine is maintained at the base of the crypt. In order to identify stem cell markers, a number of long term lineage tracing studies have been performed, and though there are differences in the architecture of small and large intestine stem cell niche, there are some similarities in some of the markers identified. For instance, steadily dividing stem cells are marked by Lgr5 expression (Barker, van Es et al. 2007) and the cells that replace the epithelium upon injury are marked by Lrig1, Bmi-1, and mTert expression (Sangiorgi and Capecchi 2008, Montgomery, Carlone et al. 2011, Tian, Biehs et al. 2011, Powell, Wang et al. 2012). There are also similarities in the signaling pathways at work between the small and large intestine to maintain the stem cell niche.

Wnt signaling

The main signaling pathway implicated in maintenance of the stem cell niche within the intestinal tract is canonical Wnt signaling (Reya and Clevers 2005, Fevr, Robine et al. 2007). The Wnt signaling pathway was first described in the 1980's and derived its name from the high degree of conservation that *Drosophila* gene *Wingless* and murine *Int-1* shared (*Wg* + *Int-1* = Wnt) (Nusse and Varmus 1982, Rijsewijk, Schuermann et al. 1987). β-Catenin is the central mediator of canonical Wnt signaling and is localized to the cell membrane at the adherens junction through its interaction with E-cadherin (Figure 1.2A) (Logan and Nusse 2004). However, whenever β-Catenin dissociates from the cell membrane and accumulates in the cytoplasm, it is targeted for proteasomal degradation through a protein degradation complex consisting of Axin, adenomatous polyposis coli (APC), casein kinase 1α (CK1α), glycogen synthase kinase 3β (GSK3β). Whenever a Wnt ligand is present, it binds to the Lrp5/6 receptor and Frizzled on the cell membrane and is thought to sequester Axin away from the degradation complex, thereby, allowing cytoplasmic levels of β-catenin to increase. β-

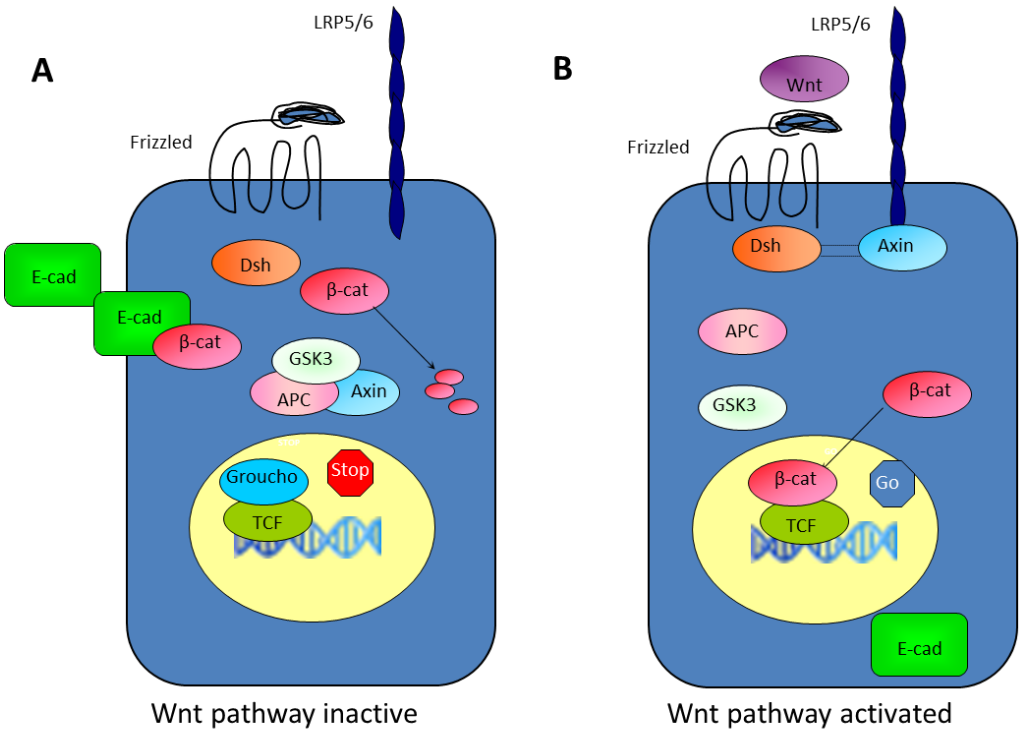


Figure 1.2. *Canonical Wnt signaling pathway.* Adapted from Logan, et al 2004. (A) Depiction of a cell when the Wnt pathway is inactive. β-catenin (β-cat) is maintained at the adherens junction on the cellular membrane by its interaction with E-cadherin (E-cad). When β-cat dissociates from the cellular membrane β-cat is targeted for degradation (A) or if β-cat dissociates in the presence of a Wnt ligand (B), β-cat translocates to the nucleus and drives a specific transcriptional program.

Gene	Function	Direct target?	Reference(s)
c-Myc	Transcription factor responding to mitogenic stimuli	Tcf/Lef binding sites within promoter	He, et al, 1998 (He, Sparks et al. 1998)
Cyclin D1	Cell cycle regulation	Tcf/Lef1 site within promoter	Shtutman, et al, 1999 (Shtutman, Zhurinsky et al. 1999)
Axin2	Negative regulator of Wnt Signaling	Tcf/Lef sit within promoter	Jho, et al, 2002(Jho, Zhang et al. 2002)
Ascl2	Proliferation in Trophoblast during development (Guillemot, Nagy et al. 1994)	Expression down with expression of Lef1 dominant negative	Jubb et al, 2006 (Jubb, Chalasani et al. 2006)
Lgr5	Unknown, co-internalizes with LRP6 on Wnt binding (Carmon, Lin et al. 2012), putative R-spondin receptor (de Lau, Barker et al. 2011)	Expression ablated upon expression of dominant negative Tcf4	Barker et al, 2007(Barker, van Es et al. 2007)

Table 1.1. *Canonical Wnt Signaling Targets.* Table displaying targets of canonical Wnt signaling, their function, and discovery.

catenin then translocates to the nucleus, displaces the transcriptional suppressor, Groucho, from T cell specific transcription factor/Lymphoid enhancer-binding factor 1 (TCF/LEF) and drives a specific transcriptional program (Figure 1.2B). Many canonical Wnt targets, genes which change expression upon activation of Wnt signaling, have been elucidated over the years (Table 1). Axin2, Cyclin D1, c-Myc, and Ascl2 have been shown to be under transcriptional control by TCF/LEF and have frequently been used as experimental markers to establish whether and where Wnt signaling is occurring within the intestinal tract in various disease states.

Within the intestinal tract, Wnt signaling is greatest toward the base of the crypt where it is required to maintain the intestinal stem cell niche. Within the small intestine, Paneth cells supply a source of Wnt ligands, predominantly Wnt3 and 11, while in the large intestine, CD24+ positive cells residing between Lgr5+ cells may also function as a source for Wnt ligand (Sato, van Es et al. 2011). In addition, Wnt signaling can be influenced by other factors such as R-spondin which functions to enhance Wnt signaling, but R-spondin cannot activate Wnt signaling on its own (Glinka, Dolde et al. 2011, de Lau, Snel et al. 2012).Mesenchymal cells surrounding the crypt have been implicated in an antagonistic role to Wnt signaling through secretion of Wnt antagonists (SFRP-1, SFRP-2, Dkk-2 and Dkk-3) ensuring that the stem cell niche is maintained toward the base of the crypt (Pinchuk, Mifflin et al. 2010). Nuclear localization of β-Catenin visualized by immunohistochemistry is also observed toward the base of the crypt (van de Wetering, Sancho et al. 2002). Active Wnt signaling is necessary for maintenance of normal intestinal homeostasis and proliferative zone (Fevr, Robine et al. 2007).

TGF-β Family Signaling

The TGF-β family of ligands is a broad series of developmentally critical growth factors and morphogens including Transforming Growth Factor βs (TGF-βs), Activins,

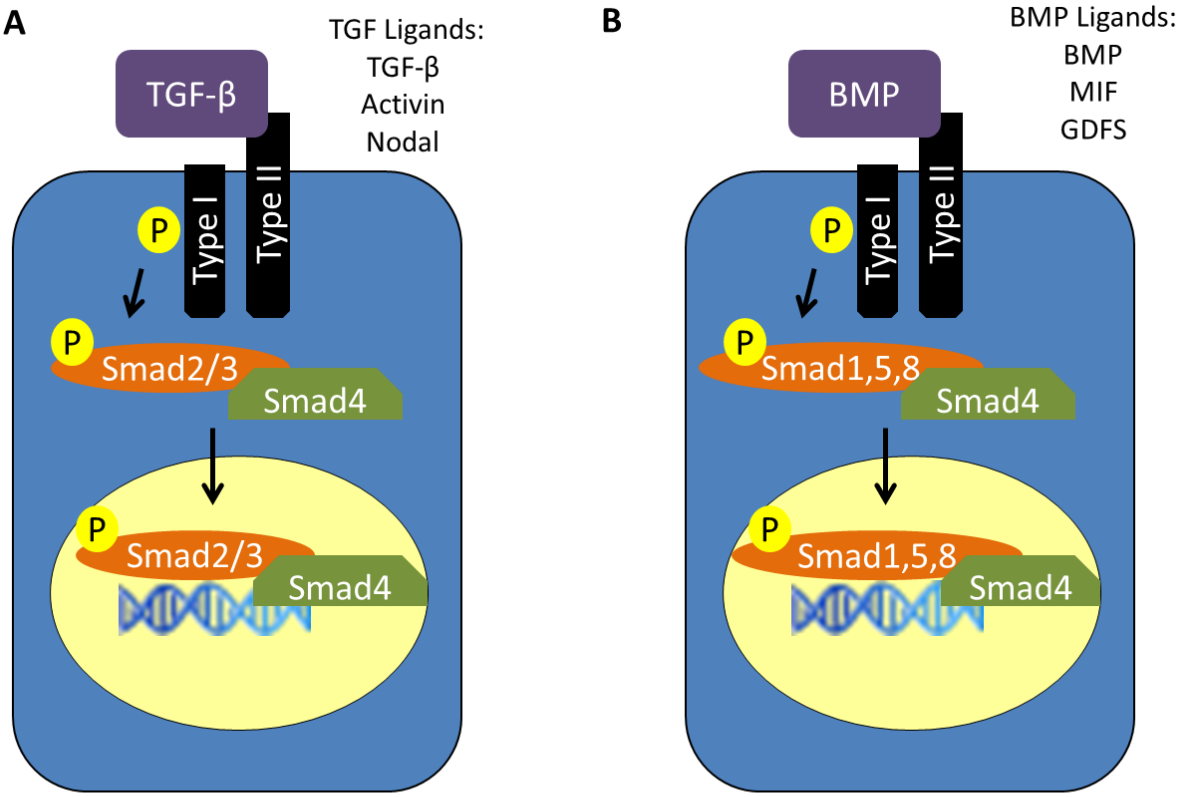


Figure 1.3. *TGF-β/BMP Signaling Pathway*. Depiction of TGF-β branch (A) of TGF- β signaling pathway showing phosphorylation of Smad2/3 which in turns binds with Smad4 prior to translocation to the nucleus. Depiction of BMP branch (B) of TGF- β signaling pathway showing phosphorylation of Smad1,5,8 which in turns binds with Smad4 prior to translocation to the nucleus. Diagrams are not to scale.

Nodals, Bone morphogenic proteins (BMPs), Growth and Differentiation Factors (GDFs), Mullerian Inhibiting Substance (MIS) (Shi and Massague 2003). For the purpose of this work, we will focus our discussion upon TGF-βs and BMPs. All major branches of TGF-β family act through ligand binding to a heteromeric complex of ligand-specific type 1 (Alk1-7) and type II serine/threonine kinase receptors (Shi and Massague 2003). Ligand binding to specific type II receptors results in formation of the heteromeric complex with type I receptors. This leads to a phosphorylation of the type I receptor, initiating a phosphorylating cascade sequence into one of two major branches of the Smad signaling pathway through phosphorylation of Receptor Associated Smads (R-Smads), which are enumerated Smads1-3, 5 and 8 (Shi and Massague 2003). Smads 1,5, and 8 function to transduce signals for the BMPs and their specific type II (BMPR-II,ActR-II/B) and type I receptors (Alk3, Alk6, and Alk2) receptors while Smads 2 and 3 transduce the signal for TGF-β through type I receptor (Alk5) and TGF-β selective type II receptor (Figure 1.3A,B). Phosphorylated R-Smads bind to the common Smad mediator, Smad4, allowing them to be efficiently translocated to the cell nucleus (Figure 1.3A,B). Smad nuclear complexes interact with specific consensus Smad DNA binding elements and other co-transcription factors regulating the expression of specific target genes, depending on the context (Massague 2008).

There are multiple levels of regulation that occur within the TGF-β signaling pathway. Ligand binding to type II receptors is regulated by a family of proteins known as ligand traps, which can specifically bind to ligands blocking their access to receptors. For example, Noggin entraps BMPs 2, 4, and 7 and inhibits activation of the type II receptors (Hirsinger, Duprez et al. 1997, Marcelino, Sciortino et al. 2001). Inhibitory Smads (Smads 6,7) inhibit signaling by interfering with the R-Smads. Smad7 can inhibit both TGF-β and BMP signaling pathway while Smad6 inhibits BMP signaling preferentially (Nakao, Afrakhte et al. 1997, Hata, Lagna et al. 1998). Ubiquitylation and

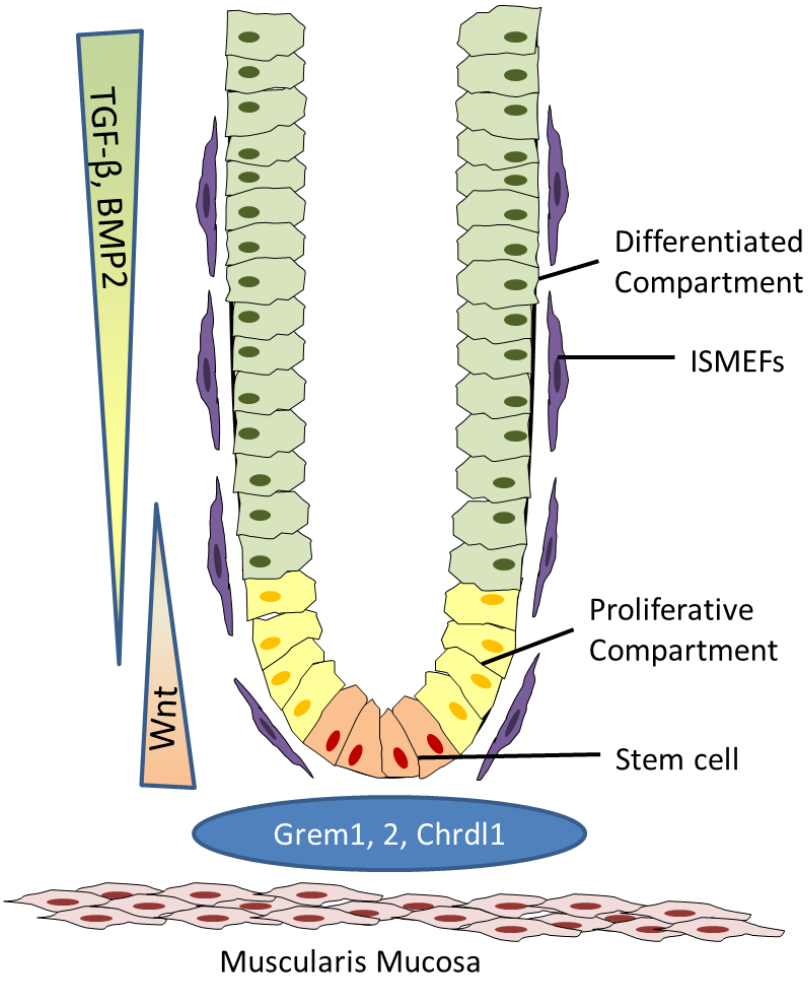


Figure 1.4. *TGF-β signaling and Wnt gradients in adult intestinal crypts.* Kosinski, *et al.,* found Smad pathway agonists, BMP1, 2, 5, 7 along with BMPR2, to be highly expressed at the top of colonic crypts by microarray analysis, while Smad pathway antagonists gremlin 1, 2 and chordin were highly expressed at the base of colonic crypts. Activation of Wnt signaling by BMP antagonists was noted by Kosinski (PNAS USA, 2007) in agreement with previous observations from others (Haramis, AP, 2004; Leung, JY, 2002; Jho, EH., 2002). Adapted with the aid of Joseph Roland.

proteasomal degradation regulate the steady-state levels of the Smad proteins (Massague, Seoane et al. 2005).

Within the epithelium of the intestinal tract, the TGF-β family of peptides inhibits mitotic activity. This activity was first noted when treatment of cultured rat intestinal epithelial cells with TGF-β resulted in inhibition of incorporation of [^{3}H]-thymidine and increased expression of sucrase suggesting both an inhibition of mitosis and promotion of functional differentiated cells (Kurokowa, Lynch et al. 1987). *In vitro* TGF-β treatment resulted in G1 cell cycle arrest, associated with down-regulation of Cyclin D1, and inhibition of Cdk4 kinase activity (Ko, Sheng et al. 1995, Ko, Yu et al. 1998). As intestinal epithelial cells migrate toward the luminal aspect of the crypt, there is increased expression of TGF-β1 and type II TGF-β receptor (Barnard, Beauchamp et al. 1989, Barnard, Warwick et al. 1993, Winesett, Ramsey et al. 1996). This expression pattern is inversely correlated with proliferation within the intestinal epithelium (Figure 1.4) (Zhang, Nanney et al. 1997). Within normal intestinal epithelium, BMP is a major driver of differentiation and apoptosis toward the luminal aspect of the intestinal crypt and is thought to inhibit Wnt signaling (Kosinski, Li et al. 2007, Brabletz, Schmalhofer et al. 2009). *In vitro* treatment with BMP-2 promotes apoptosis and growth inhibition in colon cancer cell lines (Hardwick, Van Den Brink et al. 2004). Within the intestinal crypt, antagonists to BMP signaling Grem1 and Grem2, and Chordin-like1 are expressed toward the base of the crypt (Figure 1.4) (Kosinski, Li et al. 2007), and transgenic mice expressing Noggin, a BMP antagonist, aberrantly develop crypts perpendicular to the normal crypt-villous axis (Haramis, Begthel et al. 2004). Taken together these illustrate a functional role for TGF-β signaling in normal homeostasis and differentiation within the intestinal tract.

Non-canonical TGF-β Family Signaling

Non-canonical (non-Smad) TGF-β signaling also occurs in intestinal epithelial

cells and can influence their growth and proliferation. Treatment with exogenous TGF- β can lead to activation of Erk, JNK, and p38 pathways (Yue and Mulder 2000, Derynck and Zhang 2003, Zhang 2009). Experiments using TGF-β type I receptor mutant constructs show activation of p38 (Yu, Hebert et al. 2002) and Erk pathway through activation of Ras and lead to senescence (Cipriano, Kan et al. 2011). Activation of JNK pathway by TGF-β was shown to be TAK1 and TRAF6 dependent and induced apoptosis (Shim, Xiao et al. 2005, Yamashita, Fatyol et al. 2008). Activation of Erk and Jnk can in turn lead to activation of the canonical Smad pathway through phosphorylation of R-Smads further complicating differentiating between canonical versus non-canonical effects of TGF-β treatment (Engel, McDonnell et al. 1999, Hayashida, Decaestecker et al. 2003, Hough, Radu et al. 2012, Liu, Zhang et al. 2012). Conditional knockout of BMP Receptor 1A in mice resulted in the development of intestinal polyps, inhibition of BMP signaling, resultant PTEN activation and increasing levels of pAKT in association with increased β-catenin levels (He, Zhang et al. 2004) again showing consequences of removing aspects of the TGF-β signaling pathway can lead to aberrant non-canonical signaling.

Other Signaling Pathways Implicated in Intestinal Homeostasis

The Notch signaling pathway has been implicated in cell fate decisions and differentiation (Radtke and Clevers 2005, van Es, van Gijn et al. 2005). Notch signaling functions through cleavage of the Notch receptor by proteolytic proteins upon binding to any of the Delta, Serrate, or JAG2 ligands expressed on adjacent cells (Guruharsha, Kankel et al. 2012). This releases the Notch intracellular domain (NICD) into the cytosol. NICD translocates to the nucleus and interacts with the transcription factor RBPJ to express Hes1 (VanDussen, Carulli et al. 2012) and suppresses the expression of atonal

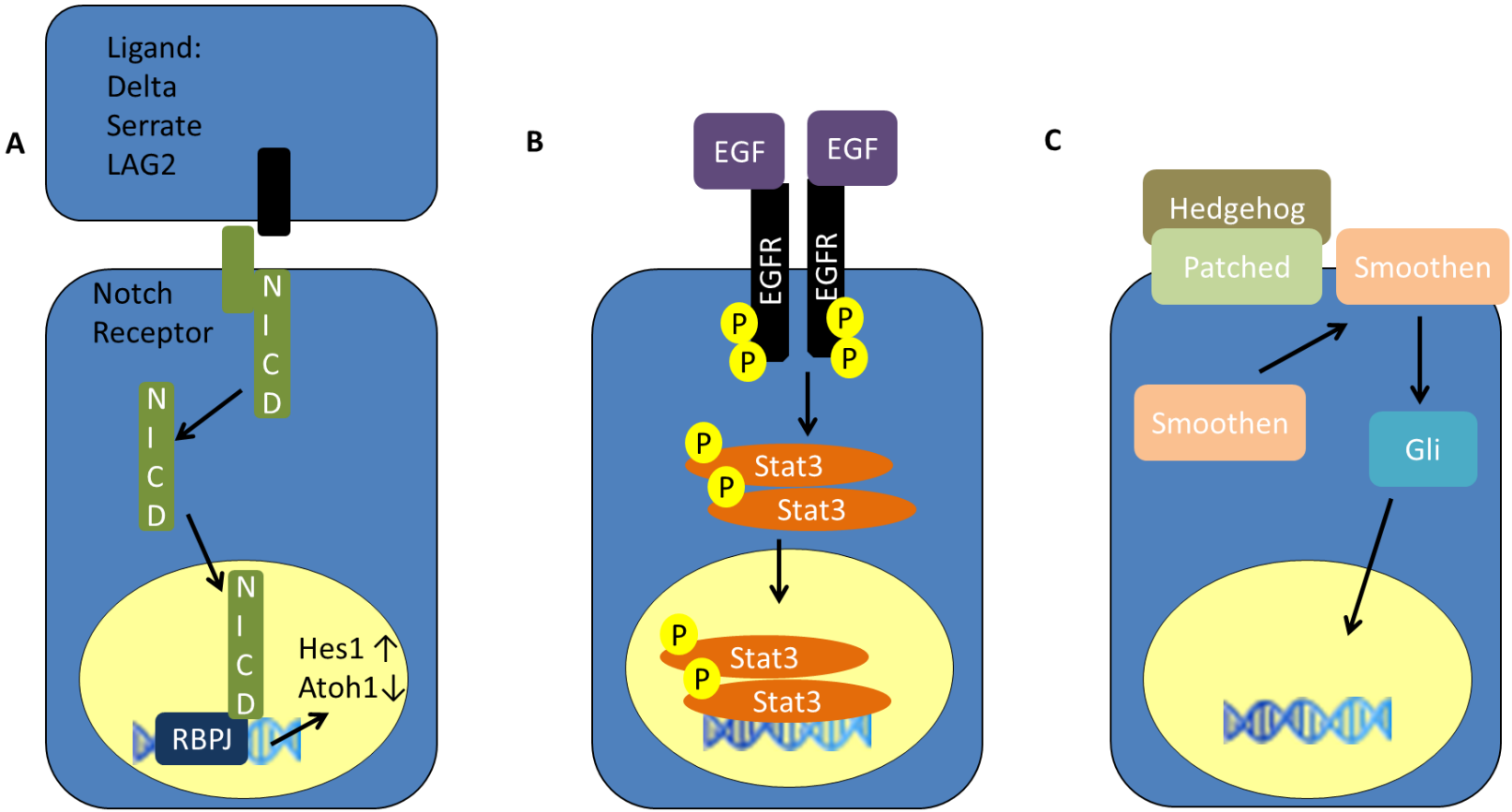


Figure 1.5. *Notch, EGF, and Hedgehog Signaling Pathways.* (A) Depiction of activation of Notch signaling in wich the Notch Intercellular Domain (NICD) is cleaved and translocates to the nucleus to bind with the RBPJ protein. Activation of Notch signaling leads to increased Hes1 expression and decreased Atoh1 expression in intestinal epithelium and leads to development into an absorptive cell (B) Depiction of EGF signaling wherein EGF ligand induces a dimerization of ErbB family of receptors (EGFR, ErbB1) to form a homodimer that leads to phosphorylation of receptors followed by phosphorylation of proteins with an SH2 binding domain (Stat3 depicted here). This permits dimerization of Stat3 and translocation to nucleus to drive proliferation. (C) Depiction of activated Hedgehog signaling wherein Hedgehog ligand binds to Patched receptor allowing Smoothen to activate Gli protein to drive transcription of target genes.

homologue 1 (Atoh1) (Yang, Bermingham et al. 2001, Shroyer, Helmrath et al. 2007, VanDussen and Samuelson 2010) (Figure 1.5A). Again, activation of Notch signaling within intestinal epithelium leads to development into absorptive enterocytes. However, in the absence of Notch activation, Atoh1 drives the differentiation down the secretory lineage (Yang, Bermingham et al. 2001) and has, for example, been shown to transcriptionally activate expression of goblet cell marker Muc2 (Park, Oh et al. 2006).

The epidermal growth factor (EGF) signaling pathway has been implicated in proliferation and maintenance of the stem cell niche (Biteau and Jasper 2011). There are four members of the ErbB receptor tyrosine kinase: Her1 (EGFR, ErbB1), Her2 (Neu, ErbB2), Her3 (ErbB3), and Her4 (ErbB4) that can combine together in homodimers or heterodimers to transduce signal upon binding to one of the eleven known ligands including EGF and transforming growth factor alpha (TGFα) (Garrett, McKern et al. 2002, Linggi and Carpenter 2006). This dimerization leads to phosphorylation of the C-terminal domain of the receptor allowing for a cascade of phosphorylation of several proteins that have an SH2 binding domain leading to activation of several downstream signaling pathways including: MAPK, PI3K, STAT, and other pathways. The end result of this activation leads to cellular growth and progression through the cell cycle (Oda, Matsuoka et al. 2005) (Figure 1.5B).

Hedgehog (Hh) signaling is mediated by a transmembrane protein Patched1 (Ptch1) which, without hedgehog ligand, suppresses another transmembrane protein Smoothen (Smo). Upon hedgehog ligand binding to Ptch1, the suppression of Smo is removed permitting Smo to activate Gliomas (Gli's) for signal transduction (Parkin and Ingham 2008) (Figure 1.5C). Though Indian hedgehog (Ihh) is expressed within intestinal epithelium, most of Hedgehog signal activation is restricted to the

mesenchyme (Kolterud, Grosse et al. 2009, van Dop, Heijmans et al. 2010). Interference with this pathway leads increased Wnt signaling and improper maturation of intestinal epithelium (Wang, Nassir et al. 2002, van den Brink, Bleuming et al. 2004). Also, mice lacking hedgehog ligand expression (Ihh and Sonic hedgehog [Shh]) display decreased thickness in the muscular layer surrounding the intestine and malformation of the enteric nervous system showing that hedgehog signal is important in intestinal development and homeostasis (Ramalho-Santos, Melton et al. 2000).

Interaction between the Wnt and TGF-β signaling pathways

As diagrammed in Figure 1.4, Wnt and TGF-β family signaling pathways occur in opposite gradients along the intestinal crypt; however, previous studies have implicated more interactions between these two pathways. As previously stated loss of BMP signaling can lead to increase pAKT levels (61) which in turn can inhibit GSK3 activity resulting in increased levels of β-catenin protein (Cross, Alessi et al. 1995, He, Zhang et al. 2004). Further, Ihh knockout mice lose mesenchymal expression of BMP ligands associated with an extension of the proliferative zone (Kosinski, Stange et al. 2010); though, a later study suggests that loss of activin expression, not BMP, is responsible for this proliferative zone expansion (Buller, Rosekrans et al. 2012). In *Xenopus*, direct interaction between the Smads and β-catenin and TCF/Lef1 have been observed within the Spemann's organizer to promote the expression of Xtwn, the *Xenopus* ortholog of human KLF5/BTEB2. KLF5 functions in the suppression of proliferation in non-transformed intestinal epithelium and is seen to be down regulated in colorectal cancer cases (Labbe, Letamendia et al. 2000, Nishita, Hashimoto et al. 2000, Bateman, Tan et al. 2004). Other investigators have reported that TGF-β3 stimulates transcription of LEF-1 that in turn interacts with Smad2 and Smad4 to inhibit E-cadherin in palate medial epithelium (Nawshad, Medici et al. 2007). Another group reported identification of a complex between β-catenin and Smad2 in alveolar epithelium upon

injury (Kim, Wei et al. 2009). However, a transcriptional regulation of β-catenin by Smad4 signaling had not been described prior to our report in 2012 (Freeman, Smith et al. 2012).

Applications of Stem Cell Biology

Enteroid Culture - Utilizing the known pathways for maintenance of the stem cell niche as well as the knowledge of the signals that are required to stimulate differentiation, researchers have developed *in vitro* culture techniques in which enteroids derived from both mouse and human intestinal crypts can be established (Sato, Vries et al. 2009). Enteroids refer to establishing three dimensional cultures using artificial extracellular matrix substrates (such as commercially available "matrigel") that permit the growth and differentiation of epithelium derived solely from the small intestine (Stelzner, Helmrath et al. 2012). Essentially, this technique depends upon isolation of intact crypt bases and the use of media containing EGF, to stimulate cells to grow and proliferate, R-spondin, a Wnt agonist to stimulate Wnt pathway activation, and Noggin, a BMP ligand trap to prevent differentiation within the culture. This technique has provided an *in vitro* avenue to study differentiation of the intestinal epithelium without contamination of the stromal component allowing for powerful analysis on dissecting how gene manipulations alter signaling in the context of epithelial homeostasis.

Colorectal Cancer – Two of the hallmarks of cancer are sustained proliferative signaling and evasion of growth repressors (Hanahan and Weinberg 2011), so it is not surprising that colorectal carcinomas have an expression pattern that resembles intestinal stem cell niche (Merlos-Suarez, Barriga et al. 2011). As discussed, activation of the Wnt pathway stimulates the proliferation within the intestinal tract, and the Wnt pathway is dysregulated in the vast majority of colorectal cancer (discussed in greater detail below). Immunohistochemical evidence of increased Wnt pathway activation as

indicated by increased nuclear localization of β-catenin has been reported at the invasive edge of colorectal cancers (Brabletz, Jung et al. 2001). Moreover, experiments using primary human colorectal cancer cells found that cells with high level of Wnt pathway activation function as the cancer stem cell (Vermeulen, De Sousa et al.). Further, stem cell markers (such as Lgr5, a target of canonical Wnt signaling) have been used to define a non-transformed intestinal stem cell gene signature that can be used to predict more aggressive disease with greater chance of recurrence in human colorectal tumors with a similar expression pattern (Merlos-Suarez, Barriga et al. 2011). Colon cancer stem cells are thought to be needed to establish metastases and represent a niche often refractory to standard therapy (Pang, Law et al. 2010, Todaro, Francipane et al. 2010). Thus, understanding the normal intestinal development and regulation of those pathways is paramount in investigating the molecular underpinnings of colorectal cancer.

Epidemiology and Pathogenesis of Sporadic Colorectal Cancer

Colorectal cancer is projected to be the third leading cause of cancer related mortality in the United States in 2014 with approximately 50,000 individuals succumbing to this disease each year and another 150,000 patients will be newly diagnosed (Siegel, Naishadham et al. 2013). The vast majority (>90%) of cases are diagnosed in patients greater than 50 years of age and are thought to occur due to gradual accumulation of mutations over time (Hawk and Levin 2005, Cress, Morris et al. 2006, Siegel, Naishadham et al. 2013). While increased surveillance by colonoscopy, greater usage of NSAIDS, and improved therapies have purportedly reduced mortality since the 1980's (Fearon 2011) , the five year survival rate for patients diagnosed with stage IV disease remains low (12.9%) (Edge and Compton 2010). While local tumor growth can lead to organ dysfunction and death, 90% of cancer related deaths are attributed to metastatic

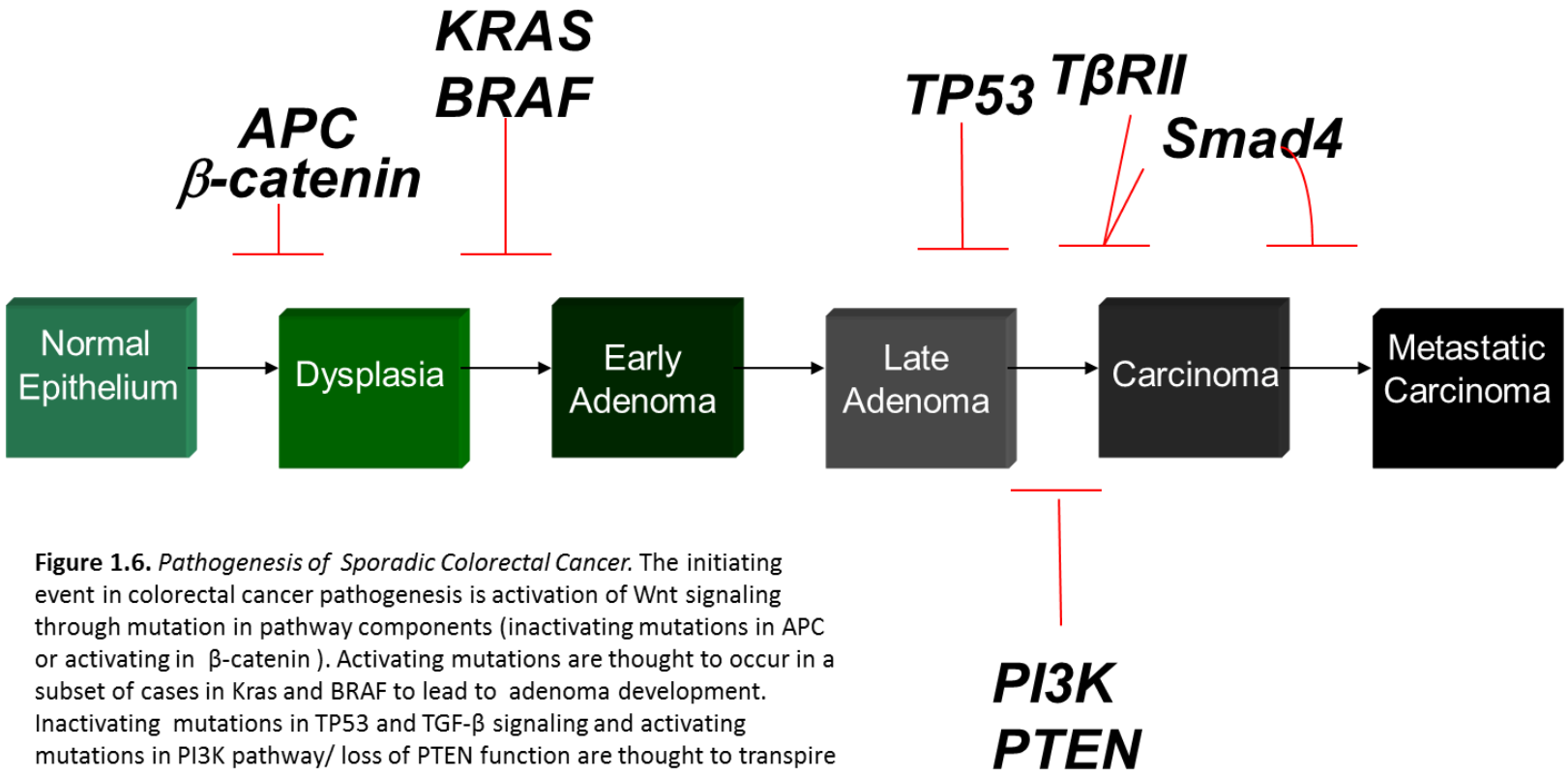


Figure 1.6. *Pathogenesis of Sporadic Colorectal Cancer.* The initiating event in colorectal cancer pathogenesis is activation of Wnt signaling through mutation in pathway components (inactivating mutations in APC or activating in β-catenin). Activating mutations are thought to occur in a subset of cases in Kras and BRAF to lead to adenoma development. Inactivating mutations in TP53 and TGF-β signaling and activating mutations in PI3K pathway/ loss of PTEN function are thought to transpire later. Adapted from Fearon, 2011.

disease (Hanahan and Weinberg 2000, Gupta and Massague 2006). The process of metastasis depends upon a cancer cell's potential to invade neighboring tissue, access vascular or lymphatic vessels, survive dissemination and extravasate to establish residence in distant organs and tissues. Therefore, understanding the signaling required and regulators of the metastatic process is essential in combating cancer mortality.

In sporadic colorectal cancer, mutations are thought to accumulate in a stochastic manner over time with the key gatekeeper mutation occurring in APC (Figure 1.6) (Fearon and Vogelstein 1990). APC functions as a suppressor of the Wnt signaling pathway via the degradation of cytoplasmic β-catenin (Figure 1. 2). Whenever APC is mutationally inactivated, inappropriate accumulation of cytoplasmic β-catenin leading to activation of the Wnt pathway within the colonic crypt occurs. Mutational inactivation of APC occurs in approximately 80% of all colorectal cancer cases (Fearon 2011) while another 5% of cases have activating mutations in β-catenin (Fearon 2011) that prevent β-catenin degradation by the proteasomal degradation complex. A small number of cancers exhibit translocations in R-spondin2 leading to overexpression and increase Wnt pathway activation (Seshagiri, Stawiski et al. 2012). All of these mutations thus lead to overexpression of Wnt targets such c-Myc and Cyclin D1 which promote cell cycle transit.

As lesions continue to accumulate mutations, activation mutations and loss of tumor suppressors within other pathways can also transpire (Figure 1.6). Mutations in Ras or BRAF occur in 30-40% of colorectal cancer cases (Bos, Fearon et al. 1987, Davies, Bignell et al. 2002, Fearon 2011) and lead to activation of mitogen activated kinase signaling pathway. Mutations in Ras are thought to occur early on in disease progression and lead to development of adenomatous lesions in the background of Wnt pathway activation (Pretlow and Pretlow 2005). TP53 mutation prevents p53's normal function in mediating cell cycle arrest and apoptosis checkpoint (Vazquez, Bond et al.

2008, Vousden and Prives 2009). Loss of heterozygosity, chromosomal deletion of a gene and surrounding area, and missense mutation of the other allele of p53 is thought to occur in up to 70% of colorectal cancer cases (Vogelstein, Fearon et al. 1988). Activation mutations in PI3K pathway occur in 25% (Samuels, Wang et al. 2004) of cases while inactivation of PTEN occurs in approximately 20% (Laurent-Puig, Cayre et al. 2009). Though the PI3K pathway is downstream of Ras signaling it is thought that activation of the pathway by Ras mutation alone may be inefficient (Li, Zhu et al. 2004). Although these pathways play key roles in the pathogenesis of colorectal cancer, in depth study of their function falls outside the scope of this work and will not be discussed.

TGF-β signaling in Colorectal Cancer

A majority of late stage colorectal cancer cases have aberrations within the TGF-β signaling pathway (Fearon 2011). Treating adenomas with exogenous TGF-β showed a similar mitotic inhibition as treating non-transformed epithelium with TGF-β. However, treatment of adenocarcinoma with TGF-β shows a decreased response to growth inhibition (Manning, Williams et al. 1991). Furthermore, a direct correlation between sensitivity to TGF-β growth inhibition and tumor cell differentiation state has been found, using human colorectal carcinoma cell lines (Hoosein, McKnight et al. 1989). Loss of this growth inhibitory response to TGF-β associates with malignant transformation in other cancer types such as breast and retinoblastoma, as well (Arteaga, Tandon et al. 1988, Kimchi, Wang et al. 1988, Sun, Wu et al. 1994).

One mechanism by which this loss of sensitivity to TGF-β treatment can occur is through down-regulation or mutation in TGF-β receptor II. Loss of function mutations in the TGF-β receptor II in colorectal cancer occur in the majority of tumors that exhibit the microsatellite instability/replication error phenotype that make up 13% of colorectal

cancer cases (Markowitz, Wang et al. 1995). This subset of colorectal cancer cases tends to have better prognosis than other types of sporadic colorectal cancers and tends to have lesions within the proximal colon (Thibodeau, Bren et al. 1993, Gryfe, Kim et al. 2000). Mutations and loss of expression of Smad signal transduction protein can also contribute to tumor development and/or progression within the gastrointestinal tract (Elliott and Blobe 2005). Smad2 mutations have been identified in less than 10% of colorectal cancers (Eppert, Scherer et al. 1996, Ohtaki, Yamaguchi et al. 2001) while Smad3 is down-regulated in 37% of gastric carcinomas (Han, Kim et al. 2004).

Loss of function mutations of Smad4 occur in 20-30% of colorectal cancer cases, much more frequently than other Smads, (Thiagalingam, Lengauer et al. 1996, Riggins, Kinzler et al. 1997) and are thought to arise in the advanced adenomatous polyp stage of tumor progression (Rowan, Halford et al. 2005, Lassmann, Weis et al. 2007). Loss of Smad4 has been associated with decreased levels of E-cadherin, development of liver metastasis and poor prognosis in Dukes C colorectal cancer patients (Miyaki, Iijima et al. 1999, Reinacher-Schick, Baldus et al. 2004, Alazzouzi, Alhopuro et al. 2005). Loss of Smad4 expression has also been associated with increased incidence of lymph node metastasis (Tanaka, Watanabe et al. 2008). Smad4 (as well as BMPR1A) germline mutation is also implicated in juvenile polyposis syndrome, a condition which predisposes patients to developing intestinal adenocarcinoma (Howe, Sayed et al. 2004). This body of work focuses on how Smad4 functions in normal intestinal homeostasis (Chapter III) and how Smad4 loss in the background of *APC* mutation affects intestinal tumorigenesis (Chapter II).

Biological Models of Colorectal Cancer

In order to recapitulate human colorectal cancer pathogenesis and disease progression, multiple mouse models of colorectal have been developed. Several models

target the *Apc* gene due to its function in Wnt signaling and its involvement in colorectal cancer pathogenesis. The first of these developed, the Apc^{Min} (Min standing for multiple intestinal neoplasia) model, came from a random mutagen screen perform on a mouse colony (Moser, Pitot et al. 1990). The result was a truncation mutant of *Apc* gene at codon 850 which resulted in the loss of regions within the protein that function in the binding of β-catenin. This truncation mutation, following loss of heterozygosity of the wild-type allele, leads to the development of approximately 100 intestinal polyps – mostly within the small intestine. Development of more sophisticated genetic tools permitted generation of the Δ716 mutant, a truncation of *Apc* at codon 716, which results in approximately 300 small intestinal polyps (Fodde, Edelmann et al. 1994), and another low penetrant variant Apc^{1638} which has truncation at codon 1638, develops 2-3 intestinal polyps by six months of age, and may closely recapitulate the truncation mutation observed in human disease (Oshima, Oshima et al. 1995, Beroud and Soussi 1996).

Homozygous mutation of Smad4 in murine models are lethal in the early embryo; however, when a truncation of *Smad4* in exon 8 was introduced on the same chromosome as a mutant $Apc^{\Delta 716}$allele, the *cis*-compound heterozygotes produced larger, though less numerous, intestinal polyps than $Apc^{\Delta 716}$ mice (Takaku, Oshima et al. 1998). Staining of the intestinal polyps confirmed a lack of *Smad4* and *Apc* expression. Unlike humans, murine *Smad4* and *Apc* are encoded on chromosome 18, implicating loss of heterozygosity for both genes in intestinal carcinoma development. While simultaneous loss of *Smad4* and *Apc* may explain the larger size of murine intestinal polyps, it is unlikely that such simultaneous loss occurs in human colorectal carcinogenesis. *Smad4* deletion in the murine T-cell compartment results in a phenocopy of human Juvenile Polyposis, a syndrome predisposing patients to colorectal cancer development, suggesting that paracrine signaling is implicated in gastrointestinal epithelium homeostasis; however, reported human patient samples show Smad4 loss

within the epithelial compartment, not within T-lymphocytes (Kim 2006). Therefore, models which more closely recapitulate the pathogenesis of human colorectal cancer are needed to elucidate the role of Smad4 signaling in colorectal carcinoma progression.

In this body of work, we developed an inducible, intestinal epithelial specific genetic loss of Smad4 in the mouse to elucidate the role of Smad4 in normal intestinal homeostasis (Chapter III) and in tumor formation (Chapter II). Our work relies heavily on a transgenic strain established by Bardeesy *et al.* in 2006 generating an inducible truncation of Smad4 driven by Cre-recombinase. For our studies, we generated both K19 Cre^{ERT2} $Smad4^{lox/lox}$ and Lrig1Cre^{ERT2} $Smad4^{lox/lox}$ (Bardeesy, Cheng et al. 2006, Means, Xu et al. 2008, Powell, Wang et al. 2012) compound transgenic mice that predictably lose expression of Smad4 specifically within the intestinal epithelium upon tamoxifen treatment. In this work, we have identified a previously unknown transcriptional regulation of the β-catenin gene (*Ctnnb1*) by Smad4 signaling (Chapters II and III) that lead to increased canonical Wnt signaling and increased tumor burden in the context of mutated *Apc* (Chapter II). In the context of non-transformed epithelium, we observed an increased proliferative zone and fewer numbers of enterocytes, goblet, and enteroendocrine cells within Smad4 null crypts compared to Smad4 expressing crypts using both K19 Cre^{ERT2} $Smad4^{lox/lox}$ and Lrig1Cre^{ERT2} $Smad4^{lox/lox}$ mouse models. We developed Smad4 null enteroids using *in vitro* tamoxifen treatment and observed no increase in canonical Wnt targets in the Smad4 null tissue. We instead observed an increase in Erk activity and preliminary results points to this regulation to be associated with non-canonical activation of TGF-β family signaling. When crossed on the Apc^{1638} background, K19 Cre^{ERT2} $Smad4^{lox/lox}$ have a ten-fold increase in tumor burden and associated increase in Wnt activity. This increase in Wnt activity is associated with increased levels of β-catenin mRNA supporting a previously unreported role for Smad4 signaling in transcriptionally regulating Wnt signaling in the intestinal tract.

Summary

Intestinal homeostasis is critical in maintaining the delicate balance between proliferation to maintain the epithelial layer that is constantly being shed and differentiation which affords the tissue its functions of nutrient absorption and being a barrier. This maintenance of homeostasis requires precise signaling to transpire along the crypt axis. Within the intestinal tract multiple pathways have been implicated in the proliferation and differentiation process including the Wnt (proliferation) and TGF-β (differentiation) signaling pathways. In Chapter III, we describe studies showing that upon losing Smad4 signaling there is an increase in the number of cells within the proliferative zone of the large intestinal crypt accompanied by a decrease in mature colonocytes. We also identify a role of Smad4 in transcriptional regulation of β-catenin though the data suggests that in the context of non-transformed epithelium that there is no detectable physiological increase in Wnt signaling. Instead, in this context, we observe an increase in Erk activation that is possibly due to non-canonical signaling involving the BMP signaling pathway.

In the pathogenesis of colorectal cancer, many tumor suppressor pathways are inactivated. Most spontaneous colorectal cancers (~80%) have mutations in APC which permits increased Wnt/ β-catenin signaling and a majority of cases has some defect in TGF-β signaling critical for development and intestinal homeostasis. In Chapter II, we explore the regulation of β-catenin by Smad4 and describe a new role of the tumor suppressor Smad4 in the transcriptional repression of β-catenin. In the context of transformed tissue, we observe an increase of canonical Wnt signaling and observe a dramatic increase in tumor burden when Smad4 expression is lost. Overall, these results provide insight into the role of Smad4 in maintaining normal intestinal homeostasis and how its loss in colorectal cancer leads to tumor progression and metastasis. By

understanding the cellular basis for these processes, we hope to eventually learn novel ways to target and reverse these effects in cases in which TGF-β/BMP mediated signaling has been loss.

CHAPTER II

SMAD4-MEDIATED SIGNALING INHIBITS INTESTINAL NEOPLASIA BY INHIBITING EXPRESSION OF β-CATENIN

Abstract

Mutational inactivation of adenomatous polyposis coli (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. Progression of CRC also involves inactivation of signaling via transforming growth factor 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 on levels of *β-catenin* messenger RNA (mRNA) and Wnt signaling. 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 transiently expressed Smad4 in SW480 colon cancer cells. We analyzed adenomas from ($APC^{\Delta 1638/+}$) and ($APC^{\Delta 1638/+}$) × ($K19Cre^{ERT2}Smad4lox/lox$) mice by using laser capture microdissection.

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* ($APC^{\Delta 1638/+}$), Smad4-deficient intestinal adenomas had

increased levels of *β-catenin* mRNA and expression of Wnt target genes compared with adenomas from $APC^{\Delta 1638/+}$ mice that expressed Smad4.Transcription of *β-catenin* is inhibited by BMP signaling to Smad4. These findings provide important information about the interaction among transforming growth factor β, BMP, and Wnt signaling pathways in progression of CRC.

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 contain adenomatous polyposis coli (APC), Axin, and GSK3β. However, in the presence of Wnt ligand, GSK3β 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 genes (Clevers 2006).

Tight regulation of Wnt signaling within the normal intestinal tract is critical for maintenance of normal intestinal stem cell function and tissue homeostasis (Clevers 2006). 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 progression of colorectal cancer (Kinzler and Vogelstein 1996). Inactivation of APC alone does not appear sufficient to fully activate Wnt signaling or to convey an invasive and metastatic behavior in tumor cells (Vermeulen, De Sousa et al. , Brabletz, Schmalhofer et al. 2009). 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 the paradigm of posttranslational regulation. In particular, a role for regulation of *β-catenin* messenger

RNA (mRNA) expression as an important mechanism to modulate Wnt signaling and progression of colon cancer has not been described.

The transforming growth factor (TGF)-β/bone morphogenetic protein (BMP)/Smad4 pathway is a developmentally crucial pathway that is also frequently mutated in colon cancer (Grady, Myeroff et al. 1999). BMP antagonists are expressed in the intestinal stem cell niche; whereas, BMP and TGF-β signaling activity increases as cells differentiate and migrate along the intestinal gland toward the intestinal lumen (He, Zhang et al. 2004, Li, He et al. 2004, Kosinski, Li et al. 2007, Perreault, Auclair et al. 2007). Studies have included the BMP antagonist Noggin as a requisite factor within media for *in vitro* culture of isolated intestinal stem cells (Sato, van Es et al. 2011). In colon cancer, TGF-β receptor type II (TβRII) is mutated in >55% of cases and BMPRI/RII is mutated in >70% of cases (Kodach, Bleuming et al. 2008); whereas, Smad4 mutations occur in 20% to 30% of cases (Miyaki, Iijima et al. 1999, Reinacher-Schick, Baldus et al. 2004). In addition, germline mutations in *Smad4* and *BMPR1A* genes are frequently found in patients with juvenile polyposis syndrome, a condition that predisposes patients to developing intestinal adenocarcinoma (Howe, Roth et al. 1998, Howe, Sayed et al. 2004). Loss of Smad4 function in the presence of *Apc* mutation in mice markedly accelerates tumor progression (Takaku, Oshima et al. 1998), 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 posttranslational 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.

Methods

Human Tissues

The tissues for microarray analysis were collected and annotated according to our established protocols approved by the institutional review boards at the Moffitt Cancer Center and Vanderbilt University as previously described (Smith, Deane et al. 2010). Tissue preparation, quality control, RNA isolation, and hybridization were conducted on the Affymetrix U133 Plus 2.0 platform (Santa Clara, CA) as previously described (Smith, Deane et al. 2010). The Gene Expression Omnibus (GEO) number associated with the data in this chapter is GSE17538.

Analysis of Gene Expression Profiles

Microarray data were normalized using the RMA algorithm in the Bioconductor package *Affy* as described (Smyth 2004); for pairwise group comparisons, *t* test in the *Limma* package(Zhang, Feng et al. 1996) in Bioconductor was used to identify differentially expressed probe sets between 2 groups under comparison (eg, normal

adjacent specimens vs stage I cancers). Student *t* test or analysis of variance was used as indicated in the figure legends. Grade A, 3′ biased annotated probes for Smad4 (Affymetrix Probeset ID 202526_at) and β-catenin (Affymetrix Probeset ID 223679_at) probes were selected to ensure full transcript expression. Correlation analysis of scatter plots was performed using GraphPad Prism 5.02 (La Jolla, CA).

Cell Culture, Transfection, and Fluorescence-Activated Cell Sorting

SW480, HCT116, and HEK293-T cells from American Type Culture Collection (ATCC) were cultured in RPMI (SW480, HCT116) and Dulbecco's modified Eagle medium (HEK293T) (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS), L-glutamine, and penicillin/streptomycin (Gibco, Carlsbad, CA). Using Effectene Transfection Reagent (Qiagen, Valencia, CA), SW480 cells were transiently transfected with 0.4-0.8μg pRK5 (empty vector) or 0.4-0.8μg pRK5-DPC4 Flag (Smad4) (Addgene, Cambridge, MA) (Shiou, Singh et al. 2007). Fluorescence-activated cell sorting was performed on SW480 cells transiently co-transfected with pRK-5 Smad4 (0.8 μg/mL) and pEGFP (1.0 μg/mL). Cells were sorted by using the FACSAria System (BD Biosciences, San Jose, CA) gated at 488nm 48 hours after transfection. The top 30% of green fluorescent protein–expressing cells were selected for RNA and protein isolation.

TOPFlash and FOPFlash reporter plasmids (Millipore, Billerica, MA) with or without siRNA were transfected and analyzed at 48 hours after transfection by using a BioTek II luminometer as previously described (Dhawan, Singh et al. 2005). FOPFlash activity did not increase with any of the reported treatments. Luciferase reagents (Dual-Glo Luciferase Assay System catalog no. E2940; Promega, Madison, WI) were used per the manufacturer's instructions. Oligonucleotide-mediated (siRNA) gene targeting was performed as follows: HEK293T or HCT116 cells at 30% confluence were co-transfected

with TOPFlash or FOPFlash, Renilla (pRL-TK, 1.0 μg/mL) plasmids, and 50 nmol/L control (Scrambled) or Smad4-specific siGENOME SMARTpool oligonucleotides (Thermo Scientific, Waltham, MA) in antibiotic-free medium containing 10% FCS for 16 hours, according to the manufacturer's instructions. Medium was changed to 10% FCS media supplemented with antibiotics. Cell lysates for Western blotting were prepared 48 hours after transfection. Conditioned Wnt3a media was obtained from L Wnt-3A cells (ATCC no. CRL-2647). Briefly, L Wnt-3A cells were brought up in high-glucose DMEM supplemented with 10% FCS, L-glutamine, penicillin/streptomycin, and G418 at 400 μg/mL. Cells were treated with a 1:1 mixture of complete media and conditioned media per ATCC protocol. A BMP-response element (BRE2-Luc, a kind gift from Dr. Peter Ten Dijke) plasmid was used to determine BMP-mediated transcriptional activity 48 hours after transfection.

Immunoblots

Cell lysates for Western blotting were prepared 48 hours after transfection unless otherwise noted. Western blotting was performed as described (Nam, Lee et al. 2010) with 10 μg of protein lysate loaded per lane. Antibodies to β-catenin (#610154; BD Biosciences, San Jose, CA), Id2 (sc-489; Santa Cruz Biotechnology, Santa Cruz, CA), Smad1 (#06-653; Upstate Cell Signaling, Lake Placid, NY), p-Smad1 (#9511; Cell Signaling, Beverly, MA), Smad2 (#51-1300; Zymed Laboratories, San Francisco, CA), p-Smad2 (#3101; Cell Signaling), Smad4 (sc-7966; Santa Cruz Biotechnology), and β-actin (#A5441; Sigma Chemical Co, St Louis, MO) were used to detect the indicated proteins.

RNA Isolation, Preparation, and Analysis

For RNA collection, cells were seeded equally into 6-well plates and harvested in parallel at 70% to 80% confluence 2 days after seeding using the Qiagen RNeasy Kit

(Valencia, CA). RNA quality was assessed based on A260/A280 between 1.8 and 2.0 and presence of intact 18S and 24S ribosomal bands by gel electrophoresis. RNA concentration was determined by UV spectrophotometry. Complementary DNA synthesis was conducted using purified 300 ng RNA and Superscript III reverse transcriptase (Roche, Basel Switzerland) according to the manufacturer's instructions. PCR amplification of complementary DNA was performed by using gene-specific primers (Universal Probe Library; Roche). The amplification protocol consisted of incubations at 95°C for 15 seconds, 60°C for 1 minute, and 72°C for 1 minute for 45 cycles by using the Light Cycler 480 (Roche). All quantitative PCR reactions were performed in quadruplicate and normalized to mock transfected samples for qPCR on HEK293T or HCT116 cells. Statistical analysis of fluorescence was performed using Light Cycler 480 SW Version 1.5 (Roche).

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation assays were performed by using the EZ-Magna ChIP Kit (catalog no. 17-408; Millipore, Billerica, MA) according to the manufacturer's instructions. Briefly, protein A beads (CS200637; Millipore, Billerica, MA) were coated with 2% bovine serum albumin overnight at 4°C. HEK293T cells were fixed in fresh 1% PFA for 10 minutes at room temperature. After cell and nuclear lysis disruption, lysates were sonicated to produce cross-linked DNA fragments that range between 200 to 1000 base pairs in size, as determined by agarose gel electrophoresis. Lysates were quantitated by using the bicinchoninic acid protein assay to assess chromatin content, and 300 µg was loaded for an overnight incubation at 4°C with RNA polymerase II antibody (5 µL; catalog no. ab5408; Abcam, Cambridge, MA), anti–acetyl histone 3 (5 µL; catalog no. 06-599B; Millipore) for positive control, or immunoglobulin G (5 µL; catalog no. PP-64B; Millipore) for negative control (controls not shown). The

immunoprecipitations were then washed and DNA isolated before qPCR analysis. DNA was subjected to qPCR with a SYBR Green PCR Kit (Applied Biosystems, Carlsbad, CA) by using the following primers: β-catenin exon 2, forward GAT TTG ATG GAG TTG GAC ATG GCC; reverse GAA GGA GCT GTG GTA GTG GCA C.

Mouse Models

Smad4$^{lox/lox}$ and K19Cre^{ERT2} mice were obtained, bred on a C57BL/6J background, and crossed (Bardeesy, Cheng et al. 2006, Means, Xu et al. 2008). APC$^{\Delta 1638/+}$ mice were crossed with K19Cre^{ERT2}Smad4$^{lox/lox}$ to produce inducible Smad4 knockout mice on APC mutant background(Fodde, Edelmann et al. 1994). The APC$^{\Delta 1638/+}$K19Cre^{ERT2}Smad4$^{lox/lox}$ mice were killed upon noting cachexia, defined as >20% body weight loss over 1 week (n = 5 for each time point, sex, and genotype). Gross determinations of polyp number in fresh tissue were verified by 2 independent observers. Organs were harvested, formalin fixed (10 hours, ~1 h/mm of tissue), and paraffin embedded. For BrdU studies, 1mL of BrdU solution (GE) was IP injected into mice 1hr prior to sacrifice. All mouse procedures were approved by the Vanderbilt University Institutional Animal Care and Use Committee, in accordance with the National Institutes of Health Guidelines for the Care and Use of Experimental Animals.

Laser Capture Microdissection

Small intestinal polyps from APC$^{\Delta 1638/+}$K19Cre^{ERT2}Smad4$^{lox/lox}$ mice treated with vehicle (n = 4) or tamoxifen (n = 4) were frozen in OTC. Frozen sections (5 μm) were cut and dehydrated before tissue isolation by using the Veritas 704 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 by using a Micro RNeasy RNA Isolation Kit (Qiagen, Valencia, CA). Isolated RNA with RNA

integrity number values >6.5 was then amplified by using the WT-Ovation Pico RNA Amplification System (NuGEN, San Carlos, CA) before qPCR analysis by using the LightCycler 480 (Roche, Basel, Switzerland) according to the manufacturer's instructions. Expression of target genes in Smad4-expressing and Smad-4 deficient polyps was normalized by using gene expression in paired normal adjacent tissue.

Immunohistochemistry

For immunohistochemistry of mouse tissues, either a 1:50 dilution of rabbit monoclonal anti-Smad4 antibody (catalog no. 1676-1; Epitomics, Burlingame, CA) or a 1:200 dilution of rabbit monoclonal anti–β-catenin antibody (catalog no. 610154; BD Biosciences, San Jose, CA) was applied to fresh-cut intestinal sections. Tissue slides were deparaffinized, antigen retrieval was performed in citrate buffer (pH 6.0) under pressure for 15 minutes, and endogenous peroxidase activity was blocked by incubating with 3% H_2O_2 for 10 minutes. The sections were stained with primary antibodies at described concentrations and developed by using DAB substrate (Vector Laboratories, Burlingame, CA). Photomicrographs were taken with a Zeiss Axioplan2 equipped a charge-coupled device color camera (Carl Zeiss Microscopy, LLC, Thornwood, NY) at described magnifications and data acquisition/analysis software (Carl Zeiss Microscopy, LLC).

For immunohistochemistry on the human tissue array, we used antibodies to Smad4 (1:50 dilution of mouse monoclonal anti-Smad4 antibody 100 μg/mL stock; catalog no. sc-7966; Santa Cruz Biotechnology, Santa Cruz, CA) and β-catenin (1:800 dilution of mouse monoclonal anti–β-catenin antibody; 250 μg/mL stock; catalog no. 610154; BD Biosciences, Sparks, MD). Antigen retrieval was performed in EDTA at 98°C in a decloaking chamber and quenched with H_2O_2. Images were captured using the Ariol SL-50 system (Leica Microsystems, San Jose, CA) with a 20× objective on a

CoolSNAP-ES charge-coupled device camera (Photometrics, Tuscon, AZ). Selected areas at original resolution are displayed. Controls for Smad4 and β-catenin staining were 12 normal adjacent colon samples and 6 adenoma samples. The cancers were independently scored (M.K.W.) in a semi-quantitative manner, and Fisher exact test was used to determine differences in proportions. Immunoreactivity intensity scores were determined as described previously (Galgano, Hampton et al. 2006).

Results

Inverse Correlation of Smad4 and β-Catenin Expression Levels in Human Colorectal Cancer

Although loss of Smad4 expression is associated with poor clinical outcomes in patients with colon cancer (Alazzouzi, Alhopuro et al. 2005), 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 data set 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 10 normal adjacent colorectal tissue specimens (Table 3.1). We observed a significant down-regulation of Smad4 expression in both early- and late-stage colorectal tumors when compared with normal colon mucosa (Figure 2.1A; *P< .0001* for all stages compared with normal [n = 10]) and significant up-regulation of β-catenin (Figure 2.1B ; P < .002 for all stages compared with 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. Although there was no significant correlation when examining all 250 cases (Figure 2.1C ; P < .09), a significant inverse correlation was observed when examining stage 1 and 2

	VMC (N=55 Colorectal adenocarcinomas)	MCC (N=195 Colorectal adenocarcinomas)	MCC - Normal (N=10 Normal adjacent colon specimens)
Mean Age (s.d.)	62.3 (14.1)	65.3 (12.9)	61.9 (16.3)
Sex (%male)	30 (54.5%)	106 (54.4%)	7 (70%)
Normal adjacent colon specimens	N/A	N/A	10
Stage I	4 (7.3%)	29 (14.9%)	2
Stage II	15 (27.3%)	61 (31.3%)	5
Stage III	19 (34.5%)	63 (32.3%)	3
Stage IV	17 (30.9%)	42 (21.5%)	0
Median follow-up (months, min-max)	50.2 (0.4-111.3)	44.97 (0.92-142.6)	N/A
Deaths	20 (36.3%)	64 (32.8%)	N/A
Caucasian (%)	50 (90.9%)	165 (84.6%)	9 (90%)
Black (%)	4 (7.3%)	11 (5.6%)	0 (0%)
Other (%)	1 (1.8%)	19 (9.8%)	1 (10%)

Table 2.1. *Human Colorectal Cancer and Normal Adjacent Specimen Microarray Dataset Demographics.* Colorectal cancer patients from Vanderbilt Medical Center (VMC, n=55), Moffitt Cancer Center (MCC, n=195) and 10 MCC normal adjacent colon specimen patients used for microarray analyses are displayed. All patients were diagnosed with colorectal adenocarcinoma and staged according to American Joint Commission on Cancer (AJCC) guidelines (stages I-IV) and the 10 normal adjacent specimens were evaluated by a pathologist and determined to contain no adenocarcinoma contribution (only normal colonic mucosa). The normal specimens were normal adjacent colon mucosa specimens from patients whose colons were resected for colon cancer. VMC 55 includes 14 patients from the University of Alabama-Birmingham Medical Center. Other in the VMC medical record implies 'not otherwise specified' and implies 'Hispanic, not otherwise specified' in the MCC medical record.

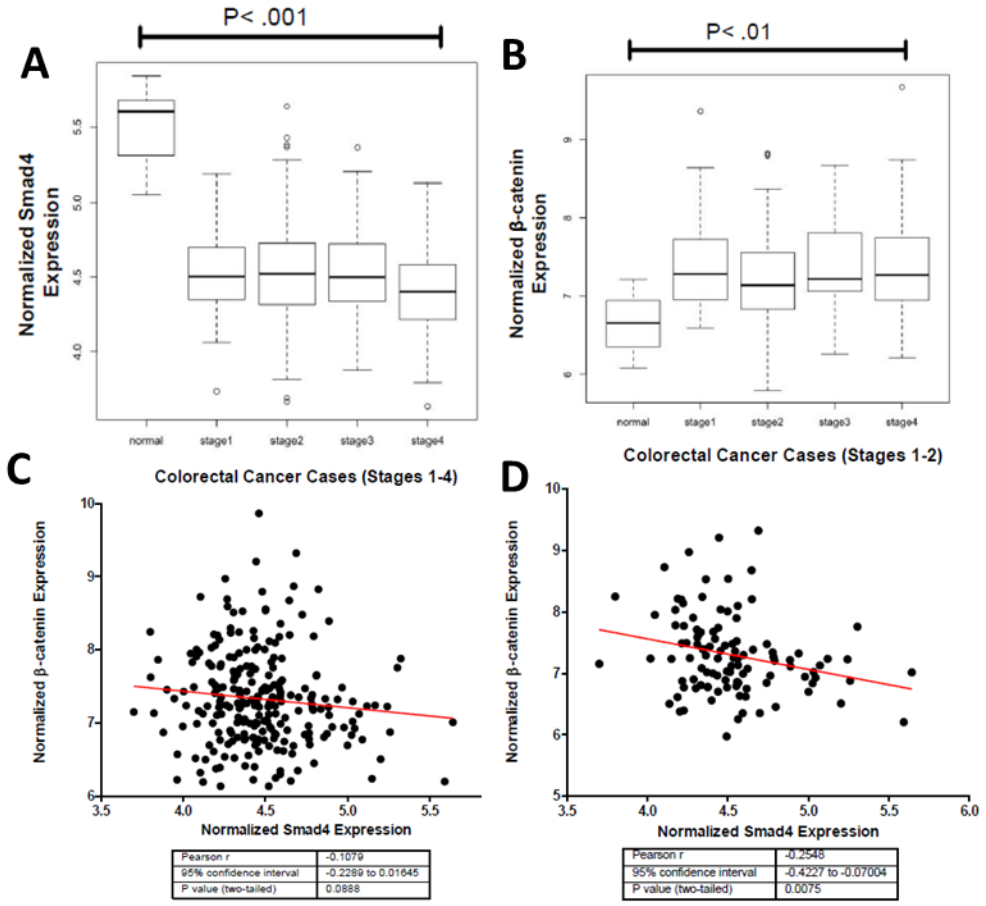


Figure 2.1. *Smad4 and β-catenin expression in human colorectal cancer.* (A and B) Microarray data of (A) Smad4 specific and (B) β-catenin mRNA levels were compared between normal and stage I-IV adenocarcinoma in a multiple comparison test. (C and D) Scatter plot displaying the normalized microarray expression values for Smad4 and β-catenin mRNA levels for (C) all stages and (D) stages 1 and 2.

cases (Figure 2.1D ; 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

Because the prevailing paradigm for regulation of β-catenin expression is posttranslational, we were surprised to find that increased β-catenin mRNA is associated with Smad4 loss in human colorectal cancer samples. We used HCT116 colon cancer cells and 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 (Morin, Sparks et al. 1997, Woodford-Richens, Rowan et al. 2001) but have mutations in *β-catenin*(Ser45 del) (Morin, Sparks et al. 1997) and *TβRII* (Markowitz, Wang et al. 1995). HEK293T cells are immortalized human embryonic kidney epithelial cells that have intact Wnt and TGF-β/BMP family signaling pathways. When compared with scrambled small interfering RNA (siRNA) treatment, Smad4 siRNA-treated HCT116 cells showed a modest increase in β-catenin protein levels (Figure 2.2A), along with a 2-fold increase in β-catenin mRNA expression (Figure 2.2B) and a 6-fold increase in TOPFlash activity (Figure 2.2C), TOPFlash is a TCF reporter plasmid that measures activation of Wnt signaling (Molenaar, vandeWetering et al. 1996). In HEK293T cells, knockdown of Smad4 expression also caused a modest increase in β-catenin protein expression (Figure 2.2D), a 4-fold increase in β-catenin mRNA levels (Figure 2.2E), and a significant increase in TOPFlash activity (Figure 2.2F). 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 was 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

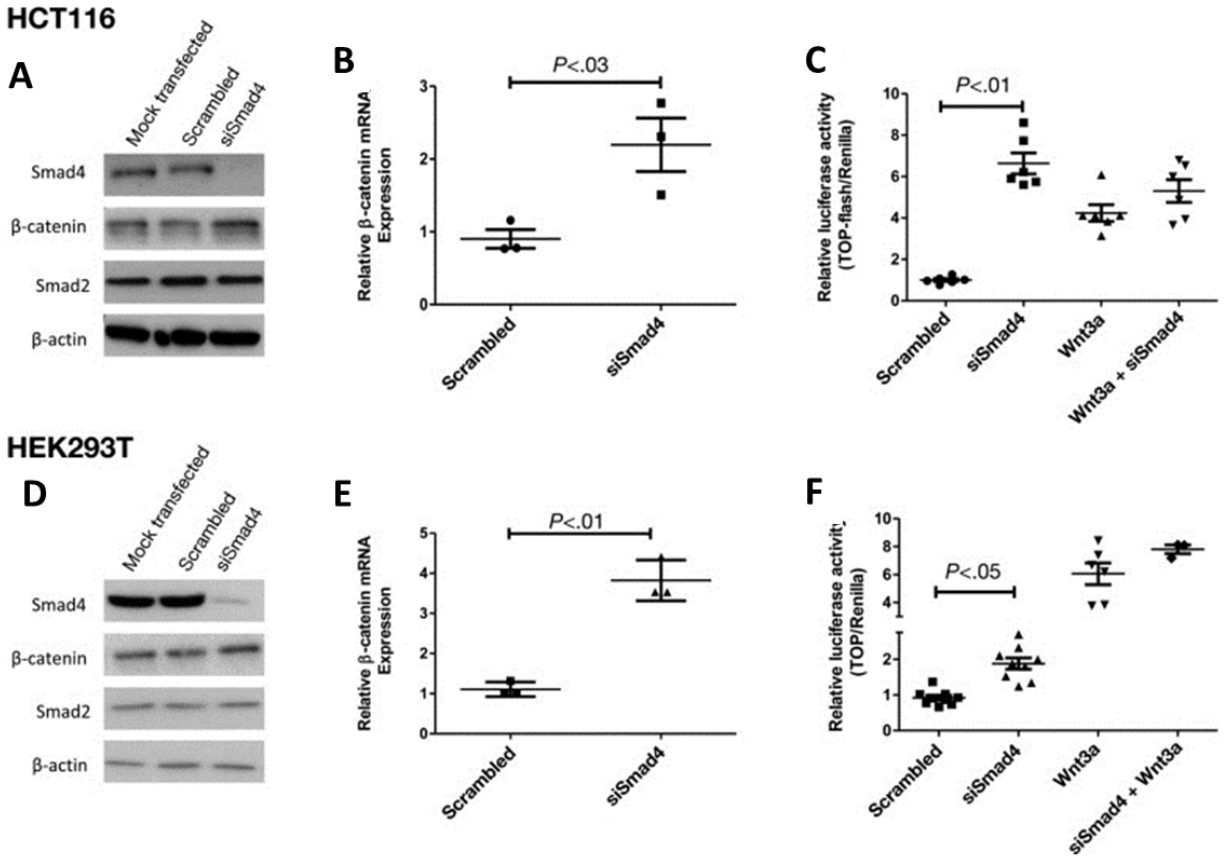


Figure 2.2. *Smad4 depletion in cultured epithelial cells results in increased β-catenin expression and activation of TOPFlash activity*. Oligonucleotide mediated Smad4 knockdown conducted in (A–C) HCT116 and (D–F) HEK293T human colon cancer cells. (A and D) Results of Western blots showing relative expression of Smad4, Smad2, β-catenin, and β-actin 24 hours after transfection with scrambled or Smad4-specific RNAi. (B and E) qPCR results showing relative expression of β-catenin mRNA expression 24 hours after 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 ± SEM displayed. Student t tests were performed to determine significance. (C and F) TOPFlash activity 24 hours after 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 was determined by ANOVA.

plasmid, providing functional evidence of nuclear β-catenin activity, in both HCT116 and HEK293T cell lines. The 2-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 a 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 allele (Woodford-Richens, Rowan et al. 2001). We previously reported that restoration of Smad4 into Smad4-deficient SW480 cells suppressed expression of the β-catenin/Wnt target Claudin-1 and that Smad4 expression also inhibited TOPFlash activity (Shiou, Singh et al. 2007). Tian et al 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 (Tian, Du et al. 2009). When Smad4 is transiently transfected into SW480 cells (Figure 2.3A), we observed a significant ($P < .02$) decrease in β-catenin mRNA and protein levels (Figure 2.3A,B) and a dose-dependent repression of TOPFlash activity (Figure 2.3C). In contrast, Smad4 did not suppress TOPFlash activity in SW480 cells when co-transfected with β-catenin driven by a heterologous promoter (Figure 2.3D). Thus, these data support a model whereby restoration of the Smad4 signaling pathway inhibits β-catenin expression, and 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

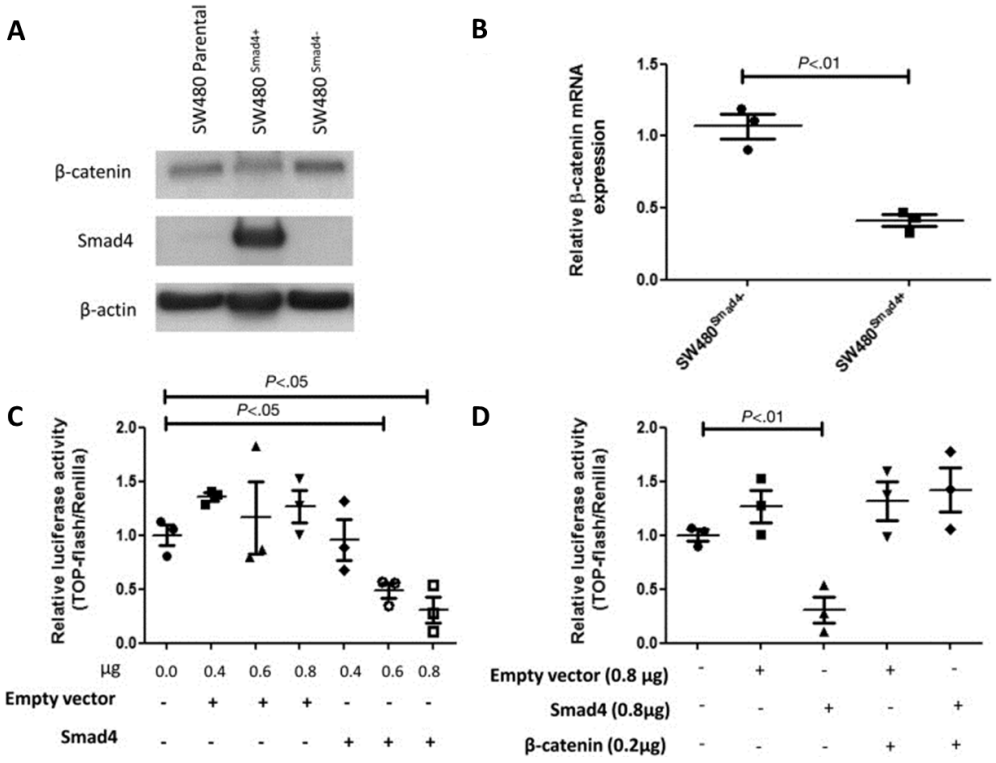
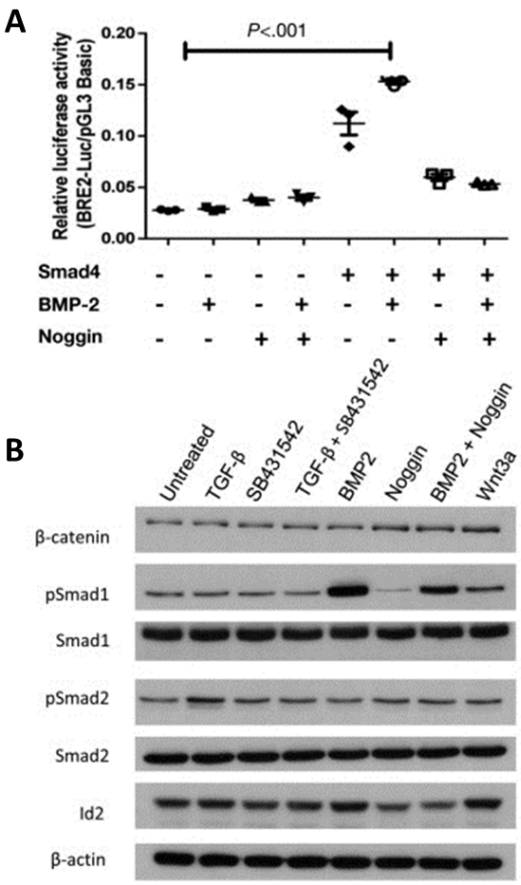


Figure 2.3. *Smad4 restoration suppresses β-catenin mRNA expression and represses TOPFlash activity in a β-catenin–dependent manner.* (A) Representative results from 3 biological replicates of fluorescence-activated cell sorted SW480 cells cotransfected with pRK-5 Smad4 (0.8 μg/mL) and pEGFP (1.0 μg/mL). 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 48 hours after transfection with p-RK5 empty vector or p-RK5-Smad4 vector as indicated. Triplicate for each condition was performed. (D) TOPFlash reporter activity in SW480 cells and in SW480 cells transiently cotransfected with 0.2 μg wild-type human β-catenin and 0.8 μg p-RK5 empty vector or 0.8 μg p-RK5-Smad4 (as indicated). Two biological replicates are displayed. Significance was determined by Student t tests. Mean ± SEM are displayed with FOP control (C and D).

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 crypt (Li, He et al. 2004). 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-Luc (ten Dijke and Korchynskyi 2002) and assessed BMP-mediated transcriptional activity associated with restoration of Smad4 expression. Previous studies have shown that SW480 and HCT116 cells express BMPR1A receptors and are responsive to BMP2 and BMP7 (Beck, Jung et al. 2006). We selected BMP2 for these experiments because the BMP2 gradient is increased toward the luminal surface of the colonic crypt (Kosinski, Li et al. 2007). BRE-Luc activity is increased with Smad4 restoration (Figure 2.4A, *lane 5*), and this activity was augmented with the addition of exogenous BMP2 ligand (Figure 2.4A, *lane 6*). 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 2.4A, *lanes 7 and 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 but remain BMP responsive (Reinacher-Schick, Baldus et al. 2004, Beck, Jung et al. 2006, Shiou, Singh et al. 2007). 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 2.4B). BMP2 treatment of HEK293T cells suppressed TOPFlash activity, but TGF-β treatment did not (Figure 2.4C). In HEK293T cells, we observed a modest but significant decrease in β-catenin mRNA levels after treatment with BMP2 and a modest



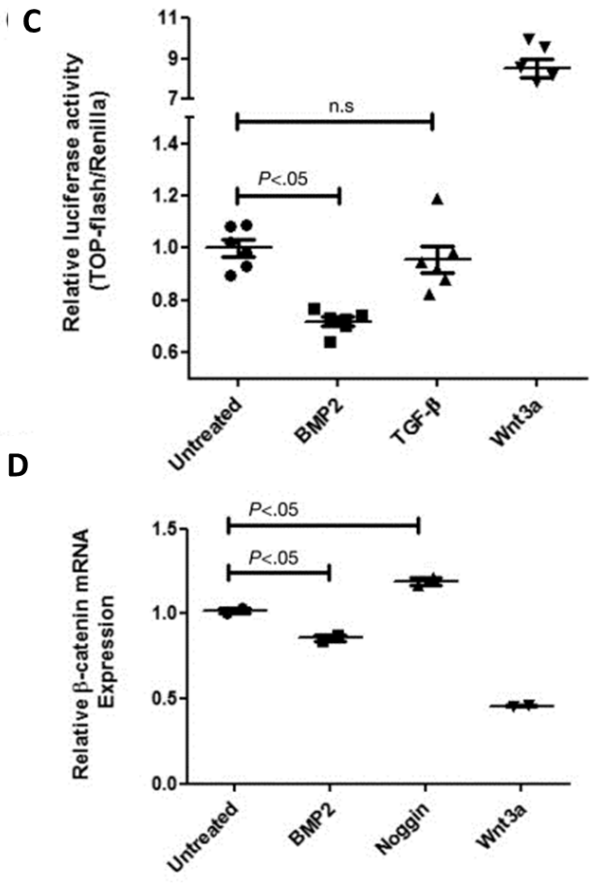


Figure 2.4. *BMP signaling suppresses TOPFlash activity and regulates β-catenin mRNA expression levels.* (A) SW480 cells were transiently cotransfected with either BRE-Luc and pCMV-Script or pCMV-Smad4 and then treated with either vehicle 20 ng/mL BMP2 and/or 200 ng/mL Noggin for 24 hours. Relative luciferase activity is graphed. Significance was determined by ANOVA. (B) Western blot of parallel experiments of HEK29T cells. Protein lysate was taken 30 minutes 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 vehicle, 200 ng/mL BMP2, 5 ng/mL TGF-β, or Wnt3a conditioned medium (1:1) and assayed for TOPFlash activity at 24 hours after transfection and 12 hours after treatment. (D) Results of qPCR assays showing relative expression of β-catenin mRNA expression in HEK293T at 12 hours after treatment with 20 ng/mL BMP2, 200 ng/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 ± SEM displayed for all. Significance was determined by Student t tests (C and D).

but significant increase in β-catenin mRNA levels after Noggin treatment (Figure 2.4D). 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 in HEK293T cells. We observed a significant decrease in RNA polymerase 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 polymerase II engagement with exon 2 of *Ctnnb1* (Figure 2.5A). To examine if this release of transcriptional repression occurs through Smad4-dependent signaling, we depleted Smad4 by using siRNA. We observed a significant increase in RNA polymerase 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 2.5B). 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.

Loss of Smad4 Promotes Carcinogenesis in the Presence of Mutated Tumor Suppressor In Vivo

To further examine the biological significance of the preceding observations, we used genetically defined conditional mouse models of Smad4 depletion. For these experiments, we crossed $Smad4^{lox/lox}$ (Bardeesy, Cheng et al. 2006) mice with $K19Cre^{ERT2}$ mice (Means, Xu et al. 2008) to generate $K19Cre^{ERT2}Smad4^{lox/lox}$. These

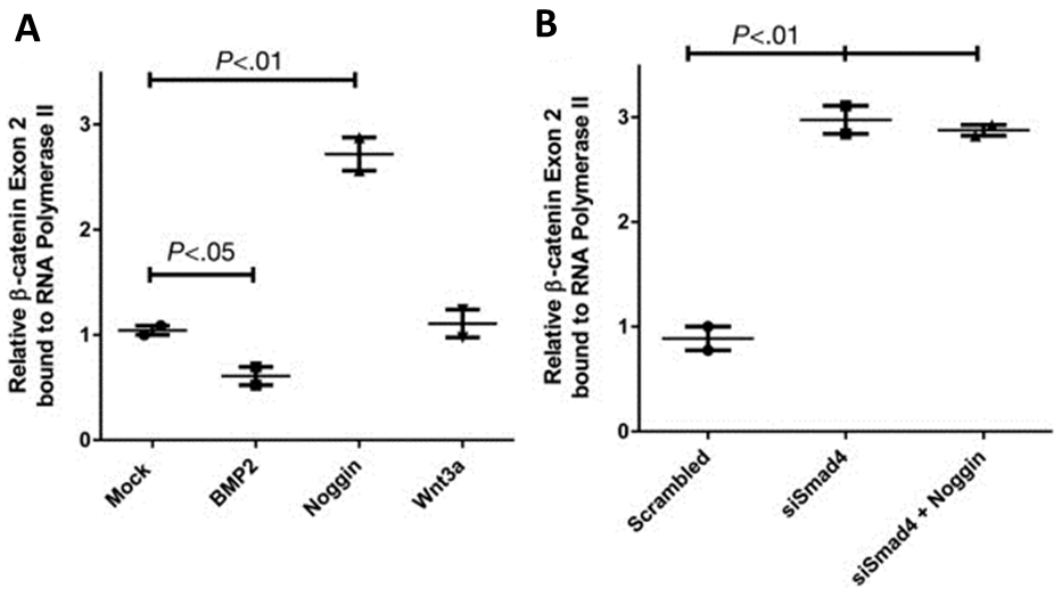


Figure 2.5. *BMP signaling regulates RNA polymerase II activity of ctnnb1*. HEK293T cells were treated for 12 hours with BMP2, Noggin, or Wnt3a, as labeled, and then nuclear lysates were prepared as described followed by immunoprecipitation with anti-RNA polymerase II antibody. (A) qPCR analysis of active RNA Pol II bound DNA sequence corresponding to exon 2 of ctnnb1 for treated samples relative to mock treated samples, as indicated. (B) HEK293T cells transfected with either scrambled siRNA or Smad4 siRNA and after 24 hours were treated with Noggin for 12 hours, as labeled. Nuclear lysates were prepared as described previously, followed by immunoprecipitation with anti-RNA polymerase II antibody and amplification of exon 2 of ctnnb1 for qPCR analysis. Results presented are from 2 biological replicates run in triplicate.

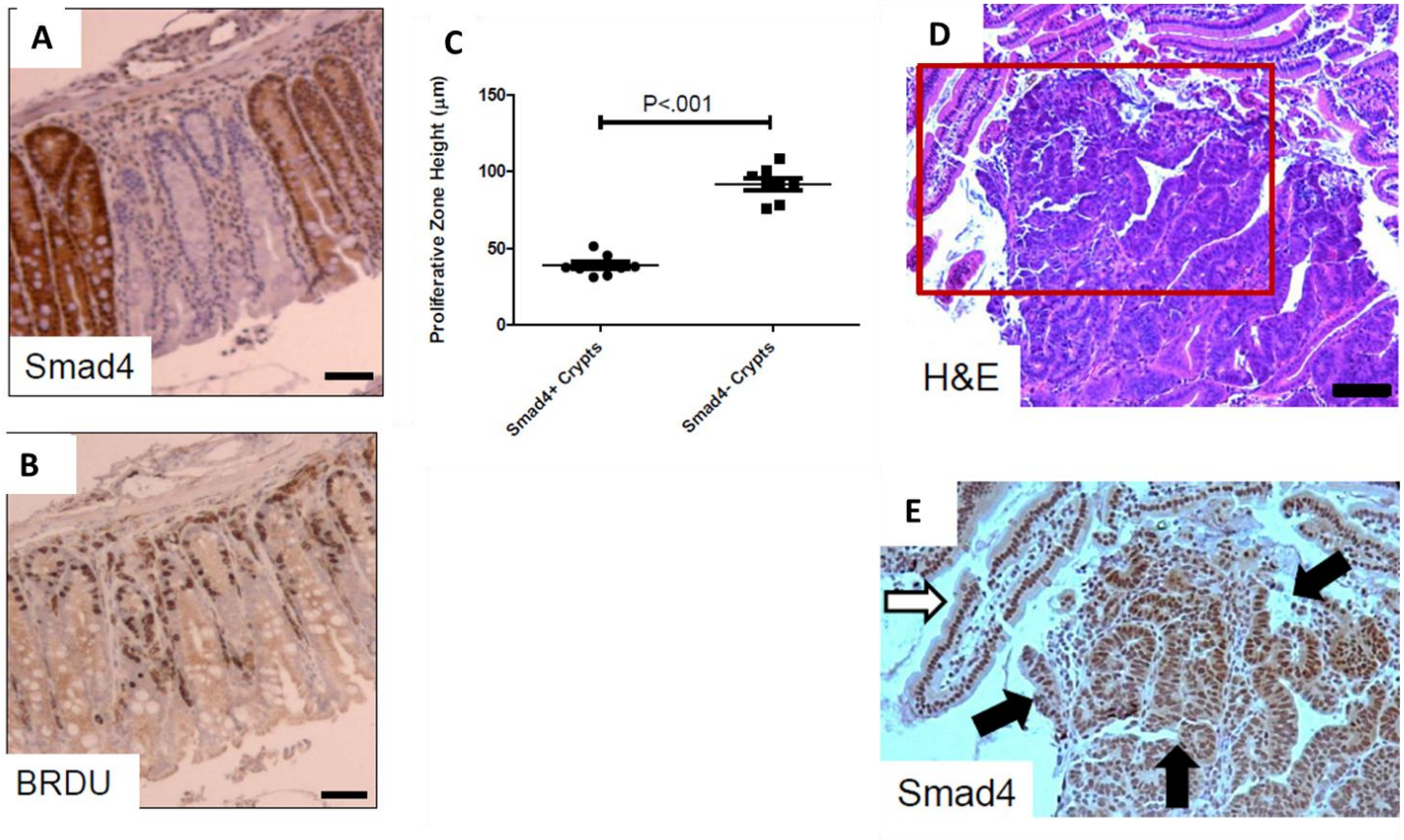


Figure 2.6. *Smad4 expression in murine models.* (A) Large intestine of K19Cre^{ERT2}Smad4lox/lox mouse 1 month after tamoxifen treatment displaying Smad4 loss. (B) Serial section of A stained for bromodeoxyuridine, indicating an expansion of the proliferative zone. Scale bars = 50 µm. (C) Quantification of bromodeoxyuridine-positive zone by height measured using ImageJ. Each data point represents an average of 2 crypts taken from mice 1 month after tamoxifen treatment (n = 8, Smad4-expressing regions; n = 8, Smad4-depleted regions). (D) H&E staining of small intestine polyp from APC$^{\Delta 1638/+}$K19Cre^{ERT2}Smad4lox/lox mouse 3 months after vehicle treatment. Scale bar = 100 µm. (E) Magnified serial section area of adenoma shown in D (indicated by red box) stained for Smad4 expression showing retention of Smad4 expression within the adenoma. White arrow indicates normal villus juxtaposed to adenomatous tissue outlined by black arrows.

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 (Figure 2.6A-C). In response to Smad4 depletion induced by tamoxifen treatment in the $APC^{\Delta 1638/+}$ background, mouse colonoscopy readily detected tumors in the colons of tamoxifen-treated mice by 1 month after treatment, whereas the colons of vehicle-treated mice appeared entirely normal (data not shown). Depletion of Smad4 resulted in a 10-fold increase in the tumor burden in both the large and small intestine by 5 weeks after tamoxifen treatment of ($K19Cre^{ERT2}Smad4^{lox/lox}$) × ($APC^{\Delta 1638/+}$) mice as compared with vehicle-treated control mice with intact Smad4 expression (Figure 2.7A, n = 4 for the vehicle and tamoxifen groups).

Immunohistochemical analysis of the polyps from tamoxifen-treated mice showed that all observed adenomatous lesions (n = 12) were depleted of Smad4 protein and exhibited abundant nuclear β-catenin staining (Figure 2.7B,C), whereas adenomas from vehicle-treated mice retained Smad4 (Figure 2.6D,E). Because ($APC^{\Delta 1638/+}$) mice rarely develop colonic adenomas (Fodde, Edelmann et al. 1994), we performed β-catenin immunostaining on the small intestinal polyps from both vehicle-treated and tamoxifen-treated ($K19Cre^{ERT2}Smad4^{lox/lox}$) × ($APC^{\Delta 1638/+}$) mice. There was a significant ($P < .01$) increase in the percentage of epithelial cells that displayed nuclear localization of β-catenin by immunohistochemistry in the tamoxifen-treated mice (Figure 2.7D and Figure 2.8; *black arrows* identify cells with nuclear β-catenin immunoreactivity, and *white arrows* identify cells lacking nuclear β-catenin immunoreactivity).

Loss of Smad4 Is Associated with Increased β-Catenin mRNA Levels and Increased Wnt Target Gene Expression in Murine Adenomas

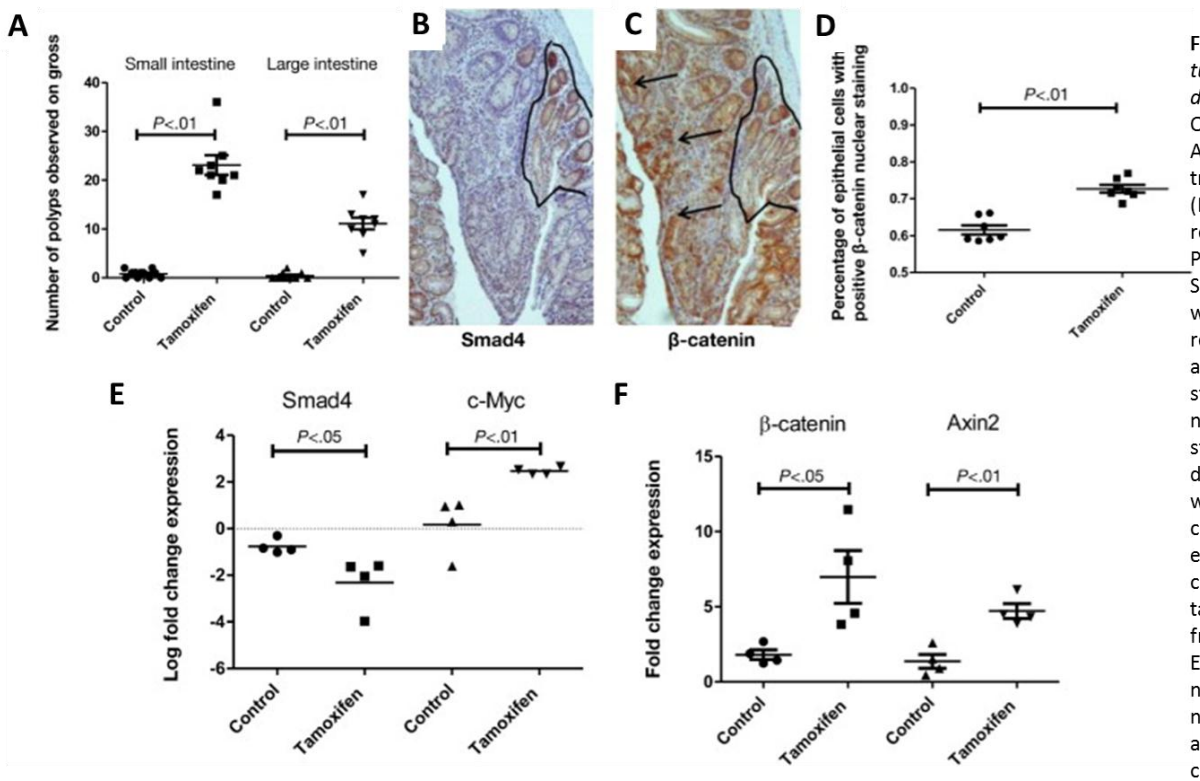


Figure 2.7. *Loss of Smad4 in vivo leads to increased tumor burden, levels of ß-catenin mRNA, and downstream targets of Wnt signaling.* (A) Quantification of polyp burden observed in $APC^{\Delta 1638/+}K19Cre^{ERT2}Smad4^{lox/lox}$ mice 5 weeks after treatment (n=8 for both treatments). Serial sections (B and C) of $APC^{\Delta 1638/+}K19Cre^{ERT2}Smad4^{lox/lox}$ polyp resected 5 weeks after treatment with tamoxifen. Photomicrographs were taken at 50x magnification. Scale bars = 200µm. (B) Smad4 immunostaining with outlined area corresponding to 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 greater than cytoplasmic staining). Each data point represents the average of positive cells within 3 high-powered fields for a single polyp (n=7 control polyps, n= 7 tamoxifen polyps). Relative expression of (E) Smad4 and c-Myc and (F) β-catenin and Axin2 mRNA in vehicle treated (n=4) or tamoxifen treated (n=4) small bowel adenomas from $APC^{\Delta 1638/+}K19Cre^{ERT2}Smad4^{lox/lox}$ mice. Expression of mRNA is displayed as fold changes normalized to the expression of target genes in normal adjacent tissue. (E) Smad4 and c-Myc data are log transformed to display the differences in a clear manner due to the magnitude of difference. Significance was determined using student's t-tests.

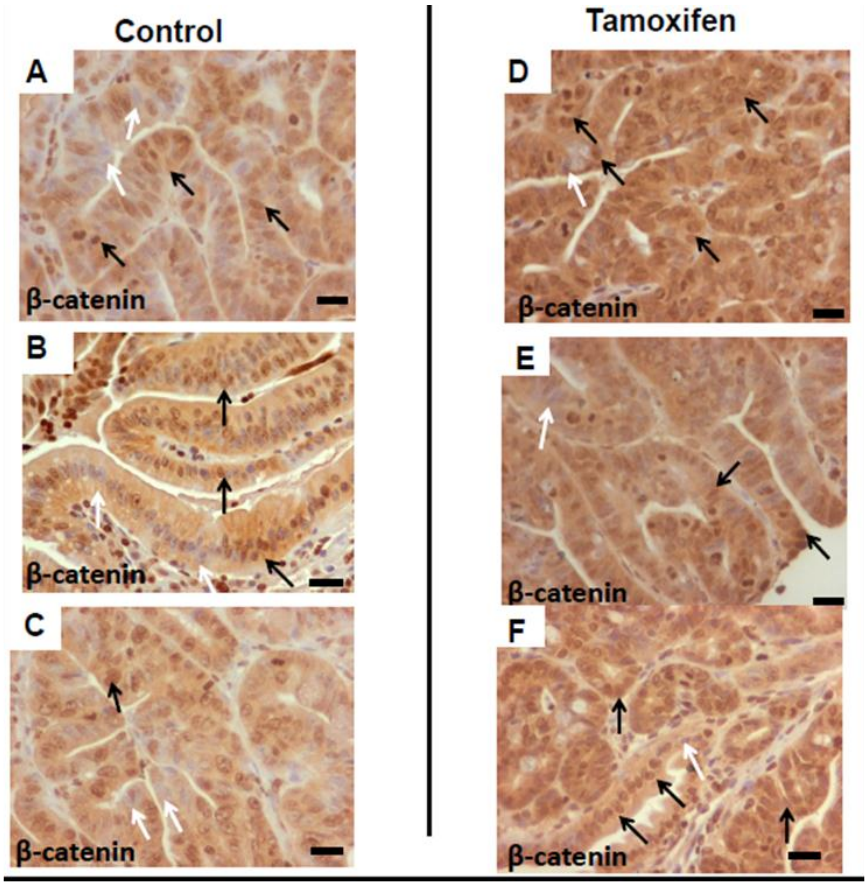


Figure 2.8. *Smad4 loss is associated with increased nuclear β-catenin staining in adenomas*. (A-F) Representative images from small intestinal adenomas from (A-C) control or (D-F) tamoxifen-treated $APC^{\Delta 1638/+}K19^{CreERT2}Smad4^{lox/lox}$ mice that have been stained for β-catenin. High-power field (original magnification 400×). Black arrows identify cells with nuclear β-catenin immunostaining, and white arrows identify cells lacking nuclear β-catenin immunostaining. Scale bars = 20 μm.

qPCR analysis of mRNA from microdissected small intestinal adenomas from vehicle-treated and tamoxifen-treated mice showed a 6-fold increase in β-catenin mRNA levels in adenomas depleted of Smad4 (Figure 2.7E) 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 with normal adjacent tissue (Figure 2.7F). This represents a significant ($P < .05$) increase in β-catenin mRNA levels when comparing lesions that have loss of Smad4 expression with those that have retained Smad4 expression. We also observed increased c-Myc (Figure 2.7E; $P < .05$) and Axin2 (Figure 2.7F; $P < .05$) mRNA expression in lesions that have loss of Smad4 expression when compared with those that retained Smad4 expression. These data indicate that loss of Smad4 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 (n = 24; demographics provided in Table 2.2). Immunostaining for Smad4 and β-catenin was scored by grading intensity as follows: 0 = negative, 1 = weak, 2 = moderate, 3 = strong. Among these samples, 19 patients (~79%) retained Smad4 expression (immunostaining score = 1–3), whereas 5 (~21%) exhibited complete tumor cell loss of Smad4 expression (immunostaining score = 0). Among those samples exhibiting complete tumor cell Smad4 loss (immunostaining score = 0), 4 (80%) had higher than median β-catenin immunohistochemical staining (immunostaining score >2) when compared with the other tumor samples. For those tumor samples retaining Smad4 expression, only 5 samples (~25%) had higher than median β-catenin immunostaining (Table 3.3). Figure 2.9 A shows a representative colon tumor section with predominant Smad4 nuclear

	VUMC TMA
Sample size	24
Mean Age (s.d.)	62 (14.58)
Sex (%male)	11 (45.8%)
Stage I	1 (4.2%)
Stage II	5 (20.8)
Stage III	7 (29.2%)
Stage IV	11 (45.8%)
Caucasian (%)	23 (95.8%)
Black (%)	1 (4.2%)
Other (%)	0
Mortality (%)	n/a

Table 2.2. *Human Colorectal Cancer Tissue Array Data and Demographics.* with colorectal cancer from Vanderbilt Medical Center (VMC, n = 24) used for tissue array analyses are displayed. All patients had a diagnosis of colorectal adenocarcinoma, which was staged according to American Joint Commission on Cancer (AJCC) guidelines (stages I-IV).

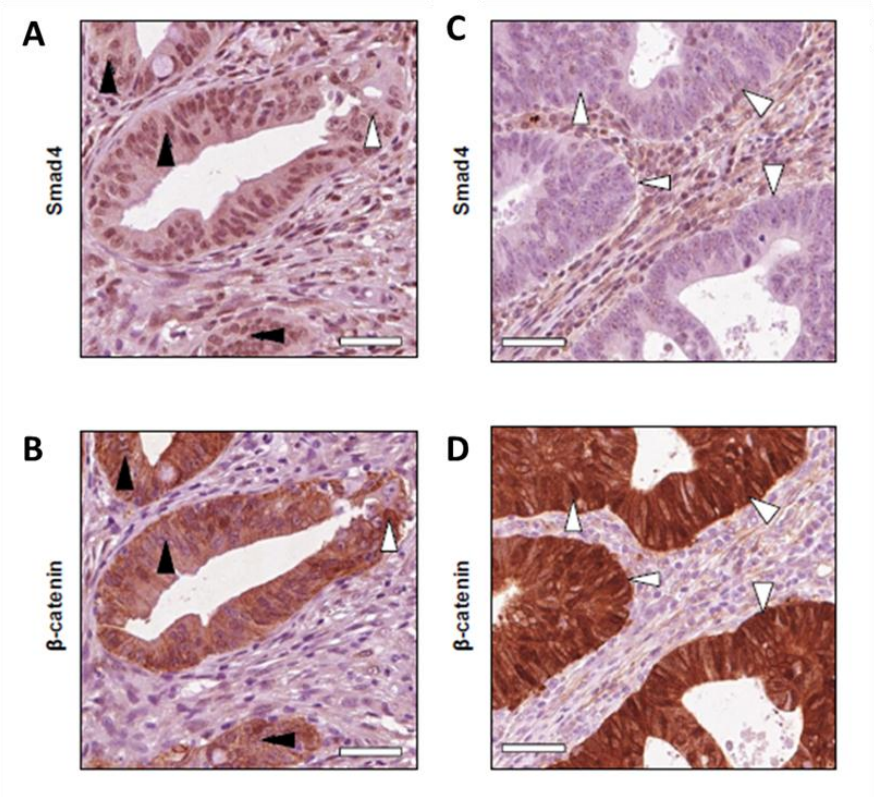


Figure 2.9. *Smad4 and β-catenin protein expression in human colorectal cancer.* (A-D) Representative patient-matched (A and B; C and D) photomicrographs of colon human adenocarcinomas (original magnification 200×) (A and C) Smad4 immunostaining and (B and D) β-catenin immunostaining. Black arrows indicate Smad4 nuclear staining, and white arrows indicate nuclei in which Smad4 was not detected. Scale bars = 50 µm.

	β-catenin levels	
	High	**Low**
Smad4 present	5	14
Smad4 absent	4	1

Fisher's Exact Test: P<.05

Table 2.3. *Smad4 and β-catenin Protein Expression are Inversely Correlated in Human Colon Cancer Specimens.* Tabulation of β-catenin protein expression as determined by immunohistochemistry in Vanderbilt University Medical Center colorectal cancer patient tissues on a tissue array (n = 24 American Joint Commission on Cancer stage I-IV cancers [Table 2.2]) was used to create a tissue array.

staining within the glandular carcinoma cells (*black arrows*). The serial section (Figure 2.9B) 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 shows Smad4 staining exclusively in stromal cells (Figure 2.9C) and strong nuclear and cytoplasmic β-catenin staining in the serial section (Figure 2.9D). Similarly, in the tumor retaining Smad4 expression, cells lacking nuclear Smad4 staining (Figure 2.9A, *white arrows*) correspond with strong nuclear and cytoplasmic β-catenin staining (Figure 2.9B, *white arrows*).

Discussion

The prevailing paradigm for regulation of β-catenin levels and Wnt signaling activity has been through the ubiquitin-proteasome posttranslational regulation of β-catenin protein stability. In this chapter, 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*. Although 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 (Shiou, Singh et al. 2007, Tian, Biehs et al. 2011). 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 patients with colorectal cancer (Fritzmann, Morkel et al. 2009). 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 (Miyaki, Iijima et al. 1999, Reinacher-Schick, Baldus et al. 2004). 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 (Alazzouzi, Alhopuro et al. 2005, Isaksson-Mettavainio, Palmqvist et al. 2006, Tanaka, Watanabe et al. 2008). In fact, based on semi-quantitative immunohistochemistry scoring, patients with stage III colorectal cancer 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 survival (Alazzouzi, Alhopuro et al. 2005). 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 BMPR1A-deficient expression (He, Zhang et al. 2004) or Noggin overexpression (Haramis, Begthel et al. 2004) 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 progression (Kinzler and Vogelstein 1996). Although loss of Smad4 expression has not been demonstrated in early intestinal adenomas associated with *Apc* mutation, Hardwick et al (Hardwick, Van den Brink et al. 2004) showed 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 posttranslational regulation of β-catenin by APC, Axin2, and GSK3β. For example, He et al implicated BMP-mediated inhibition of Wnt activity through activation of PTEN, with inhibition of PI3 kinase and Akt activity. Blockade of BMP signaling resulted in inactivation of PTEN, activation of Akt with resultant inactivation of GSK3β, and accumulation of β-catenin (He, Zhang et al. 2004). Kosinski et al (Kosinski, Li et al. 2007) reported that the BMP inhibitor Gremlin1 activates Wnt/β-catenin signaling in normal intestinal epithelial cells (IEC-18). Although intriguing, the non-canonical pathways implicated by He et al (He, Zhang et al. 2004) and Kosinski et al (Kosinski, Li et al. 2007) 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 target (Yang, Wang et al. 2009) *c-Myc* without increasing Akt activity or GSK-3β phosphorylation. This is consistent with our observations but is in contrast to the observations by He et al. (He, Zhang et al. 2004) 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 (Li, Huang et al. 2011). 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 2-hit hypothesis for carcinogenesis as previously described by Knudson (Knudson 1971). Evidence put forth by Takaku et al (Takaku, Oshima et al. 1998) showed 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% to 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 $APC^{\Delta 1638/+}K19Cre^{ERT2}Smad4^{lox/lox}$ mice. 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* (Costantini, Jho et al. 2002) 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 (Kinzler, He et al. 1998, Hoffmann and Lim 2006).

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 nuclear factor κB, EGR1, SP1, and AP1 in the proximal β-catenin promoter have been reported (Nollet, Berx et al. 1996), and we have noted 12 potential consensus Smad binding elements at positions 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 (Alazzouzi, Alhopuro et al. 2005, Isaksson-Mettavainio, Palmqvist et al. 2006). Further investigation into the precise mechanisms underlying our observations may provide new avenues to identify therapeutic targets.

CHAPTER III

LOSS OF SMAD4 IN INTESTINAL EPITHELIUM RESULTS IN DISRUPTED INTESTINAL HOMEOSTASIS AND IS ASSOCIATED WITH ACTIVATION OF ERK SIGNALING

Abstract

Inactivation of the TGF-β/BMP/Smad4 pathway occurs in 50-75% of all colorectal cancer cases, and Smad4, the central mediator of TGF-β family of signaling, down-regulation occurs in >50% of stage III patients. Despite evidence supporting TGF-β and BMP signaling as inhibitors of intestinal epithelial cell proliferation, more remains to be elucidated concerning Smad4 signaling within normal intestinal homeostasis. We utilized two mouse models with an inducible, intestine-specific recombination of Smad4 in the adult animal - K19Cre^{ERT2}Smad4$^{lox/lox}$ and Lrig1Cre^{ERT2}Smad4$^{lox/lox}$ - to induce recombination at the Smad4 locus in the intestinal stem cell compartment upon tamoxifen treatment. Intestines from sacrificed mice were harvested for enteroid isolation, Ussing chamber studies, RNA-seq analysis and preservation by formalin fixation and paraffin embedding (FFPE). We confirmed the Smad4 status via immunohistochemistry and qPCR and stained FFPE sections for different cell populations within the crypt. To determine the role for Smad4 in intestinal response to injury, we also treated control and tamoxifen treated animals with dextran sodium sulfate (DSS). Smad4 null crypts exhibited an expansion of the Ki67 positive cells in the proliferative zone and fewer Carbonic anhydrase II staining cells indicating fewer mature enterocytes. We found that loss of Smad4 in the intestinal crypt is associated with increased intestinal permeability as measured by low molecular weight FITC Dextran using the Ussing chamber. Guided by our RNA-Seq analysis, we observe increased

levels of pErk in Smad4 deplete tissue. We also observed that mice with depletion of Smad4 developed advanced mucinous adenocarcinoma after recovery from an inflammatory stimulus induced by DSS. These results suggest that Smad4 loss disrupts intestinal homeostasis by enabling persistently high levels of Erk signaling to interfere with cell maturation and is associated with loss of Cdx2 expression.

Introduction

Proper regulation of Wnt and BMP pathways is essential for intestinal epithelium homeostasis and occurs in opposite gradients along intestinal crypts (He, Zhang et al. 2004). BMP signaling is thought to inhibit Wnt signaling, driving differentiation toward the luminal aspect of the crypt (Brabletz, Schmalhofer et al. 2009). BMP antagonists are expressed toward the base of the crypt, and transgenic mice expressing Noggin, a BMP antagonist, develop crypts perpendicular to the normal crypt-villous axis (Haramis, Begthel et al. 2004). Conditional knockout of BMPR1A in mice resulted in the development of intestinal polyps, inhibition of BMP signaling, PTEN activation, and increased levels of pAKT in association with increased β-catenin levels. In Chapter II, we reported that β-catenin mRNA expression is inhibited by BMP-mediated Smad4 signaling, and loss of Smad4 signaling results in biologically significant amplification of the Wnt/β-catenin signal in the presence of an *APC* mutation. Further, we reported in Chapter II that Smad4 loss in a tumor-prone murine model resulted in increased tumor burden and increased levels of β-catenin mRNA with associated increases in canonical Wnt signaling targets (Freeman, Smith et al. 2012).

Loss of function mutations and deletions of Smad4, a transcription factor which is the central mediator of TGFβ superfamily signaling (including BMP signaling), occur in 20-30% of colorectal cancer cases and is thought to arise late in the pathogenesis of this

disease (Rowan, Halford et al. 2005, Lassmann, Weis et al. 2007). Loss of Smad4 has been associated with decreased levels of E-cadherin, liver metastasis and poor prognosis in Dukes C colorectal cancer patients (Miyaki, Iijima et al. 1999, Reinacher-Schick, Baldus et al. 2004, Alazzouzi, Alhopuro et al. 2005). In the context of normal intestinal development, the precise mechanism of how Smad4/ TGFβ superfamily signaling regulates normal intestinal homeostasis is incompletely understood. Previous reports have examined gene expression along the crypt axis and suggest that Smad4 activity correlates with differentiation but its function has not been examined *in vivo* (Kosinski, Li et al. 2007). In this chapter, we observe that Smad4 loss in the context of normal, non-transformed intestinal epithelium results in loss of epithelial homeostasis. Though we observe an increase in β-catenin mRNA levels, this is not associated with activation of the canonical Wnt pathway but with activation of Erk signaling. Following colonic mucosal injury, we note that Smad4 deficient mice develop mucinous adenocarcinomas without the need for germline mutation of *Apc* or addition of a mutagen such as AOM. We postulate that dysregulation of Erk signaling may lead to the development of intestinal carcinoma and may provide a therapeutic target in patients which have mutations in the TGF-β/BMP/Smad signaling pathway.

Methods

Mouse Models

Smad4$^{lox/lox}$, K19Cre^{ERT2} and Lrig1Cre^{ERT2} (Bardeesy, Cheng et al. 2006, Means, Xu et al. 2008, Powell, Wang et al. 2012) were obtained, bred on a C57BL/6J background, and crossed. These K19Cre^{ERT2}Smad4$^{lox/lox}$ that were then treated (via intra-peritoneal [IP] injection) with vehicle (corn oil) or tamoxifen (0.5-1mg/20g mouse). Organs were harvested, formalin fixed (10hrs, approximately ~1hr/mm of tissue) and paraffin embedded. For antibiotic treatments, mice were treated with a cocktail 1.0g/L Ampicillin,

1.0g/L Enroflaxacin, 1.0g/L Metronidazole, 1.0g/L Neomycin, 0.1g/L Vancomycin, and 30g/L Sucrose for two weeks prior to sacrifice. All mouse procedures were approved by the Vanderbilt University Division of Animal Care, in accordance with the NIH guidelines for the Care and Use of Experimental Animals.

Intestinal Permeability

Ussing Chamber Analysis

To evaluate the relationship between Smad4 loss and intestinal permeability Ussing chamber transmucosal impedance analysis and low molecular weight FITC-Dextran (3-5kDa) permeability assessment were performed as previously described (Clarke 2009). Briefly, the distal centimeter of colon was harvested and mounted on chamber units. Tissues were maintained in a water bath (42^{o}C) (NESLAB EX7, Thermo Fisher Scientific, Newington, NH)) and monitored by multichannel voltage/current clamp VCC MC8 (Physiologic Instrument of Medica Research, San Diego, CA). FITC-dextran (80mg/mL) was added to the luminal aspect of the tissue mount and 200uL samples were taken from the serosal aspect every ten minutes making sure to replace volume of samples to ensure no pressure gradient formed. Samples were read at 480nm and 520nm on a BioTek (Winooski, Vermont) fluorometer.

In vivo Studies

Mice were anaesthetized using isoflurane until non-responsive to foot pad stimulation. FITC-dextran solution (100µL, 80mg/mL) was delivered via enema, and mice were inverted for 30 min. During the procedure blood was harvested at 0 min and 30 min time points via cardiac puncture. Samples were read at 480nm and 520nm on a BioTek (Winooski, Vermont) fluorometer.

Enteroid Culture

Enteroids were derived from the K19Cre^{ERT2} Smad4$^{lox/lox}$ by using an adapted method as described in Fuller et al, 2013 (Fuller, Faulk et al. 2013). Briefly, duodenum and proximal jejunum from approximately two month old mice were harvested and incubated in an 1mM EDTA prior to spin down and plating in Matrigel (BD Bioscience) supplemented with EGF (50ng/mL), Noggin 100ng/mL, R-spondin (250ng/mL) and Wnt3a (10ng/mL). Each well was overlaid with DMEM/F12 (GIBCO, Carlsbad, CA) supplemented with HEPES 10mM), N2 (1:100), B27 (1:50), L-glutamine (1:100) and PenStrep (1:100). *In vitro* recombination was accomplished by adding 1mg/mL 4-hydroxytamoxifen (Sigma, St. Louis) to culture media and selecting enteroids which were null for Smad4 expression.

Immunostaining

For Immunohistochemistry (IHC) of mouse tissues and to avoid mouse on mouse non-specific staining, a 1:200 dilution of rabbit monoclonal anti-Smad4 antibody (Epitomics, Burlingame, CA), a 1:2000 dilution of rabbit polyclonal anti-Ki67 antibody (Abcam, ab15580, Cambridge, MA) or a 1: 2000 dilution of rabbit monoclonal anti-Cdx2 antibody (Abcam, ab76541, Cambridge, MA) were used to stain sections from resected intestinal tissue. Tissue slides were deparaffinized and endogenous peroxidase activity was blocked by incubating with 3% H_2O_2 for ten minutes. The sections were stained with primary antibodies at described concentrations and developed using DAB substrate (Vector, Burlingame, CA). Photomicrographs were taken with a Zeiss Axioplan2 equipped a CCD color camera (Zeiss AxioCam HR) and data acquisition/analysis software (Axiovision).

In Situ Hybridization

In situ hybridization for Ascl2 was performed on 8 µm paraffin sections as previously described (Gregorieff and Clevers 2010). Briefly, sections were rehydrated, digested in proteinase K (30ug/ml), post-fixed and hybridized at 65°C for 60 hrs with anti-sense Ascl2 probes diluted in hybridization solution: 5 × Saline Sodium Citrate (SSC) (pH 4.5), 50% formamide, 2% blocking powder (Roche), 5 mM EDTA, 1µg/ml yeast tRNA, and 50 µg/ml heparin. Sections were washed as follows: 2 × SSC/50% formamide for 3 × 20 min at 60°C. Sections were then rinsed in MABT buffer for 5 times for 1 min each followed by incubation in blocking buffer (MABT plus 10% heat inactivated goat serum and 1% blocking powder) for 1 hour at room temperature. Next, sections were incubated with preabsorbed alkaline phosphatase-conjugated anti-digoxigenin antibody (1/1000 dilution) (Roche) which was diluted in blocking solution overnight at 4°C. Sections were washed several times in MABT and incubated with NBT/BCIP (Roche) working solution which was diluted in NTM buffer (0.1M Tris Cl, pH9.5, 0.1M NaCl and 0.05M MgCl2) at room temperature in a humidified chamber until the color develops. Sections were rinsed twice in PBS, dehydrated and covered with coverslips with mounting medium. The plasmid carrying Ascl2 (Clone IMAGE:4163833) was used as a template DNA for RNA probe synthesis.

DSS Treatment

$Smad4^{lox/lox}$ mice and $K19Cre^{ERT2}Smad4^{lox/lox}$ mice were treated with vehicle or tamoxifen as described in *Mouse Models* section. One week after treatment, mice were subjected to three rounds of DSS exposure (5 days of 2.5% DSS water followed by 7 days of normal water).

RNA Isolation, Preparation, and RNA-Seq Analysis

For RNA collection, cells were seeded equally into 6-well plates and cells were harvested in parallel at 70-80% confluence two days after seeding using the Qiagen

RNeasy® kit (Valencia, CA). RNA quality and concentration was assessed by agarose gel electrophoresis and by UV spectrophotometry. cDNA synthesis was conducted using purified 300ng RNA and Superscript III reverse transcriptase (Roche, Indianapolis, IN) according to the manufacturer's instructions. Quantitative PCR amplification of cDNA was carried out using gene-specific primers (Universal Probe Library, Roche, Indianapolis, IN). The amplification protocol consisted of incubations at 95°C for 15s, 60°C for 1 min, and 72°C for 1 min for 45 cycles using the Light Cycler 480 (Roche, Indianapolis, IN) with continuous monitoring of fluorescence to determine Cp values. All quantitative PCR reactions were done in triplicate. Statistical analysis of fluorescence was performed using Light Cycler 480 SW Version 1.5 (Roche, Indianapolis, IN). mRNA capture, cDNA conversion, sizing, and library construction were performed using kits from the Illumina Company and by following the manufacture's recommended procedures. For RNA-Seq application, individual libraries were constructed for 3 - Smad4 wild-type enteroids, 3 - Smad4 knockout enteroids, 4 - Smad4 wild-type colonic epithelial fractions, and 4 - Smad4 knockout colonic epithelial fractions. Each library was loaded into a single lane of the Illumina Genome Analyzer II flow cell. We performed paired-end sequencing; while for pooled sample, we performed single-end sequencing. Image analysis and base-calling were performed by the Genome Analyzer Pipeline version 2.0 with default parameters. Library construction and RNA sequencing was performed in the VANTAGE Core in Vanderbilt University. After obtaining the short reads, we performed a series of quality checks, including quality score evaluation using program HTSeq and marking duplicate reads by using software SAMTools. All reads were independently aligned to a single reference file consisting of all human transcripts and the human genome in the UCSC genome assembly hg18 (NCBI build 36.1) by using TopHat (Version 1.0.10).

Immunoblots

Cell lysates for Western blotting were prepared 48 hours after transfection unless otherwise noted. Western blotting was performed as described (Nam, Lee et al. 2010) with 10 µg of protein lysate loaded per lane. Antibodies to pERK (4376-S; Cell Signal, Danvers, MA), Smad4 (sc-7966; Santa Cruz Biotechnology, Dallas, TX), and β-actin (#A5441; Sigma Chemical Co, St Louis, MO) were used to detect proteins.

Results

Smad4 Loss Leads to Altered Colonic Cell Populations

In order to understand the function of Smad4 in the context of normal intestinal homeostasis, we generated an inducible, tissue specific knockout of Smad4 (Freeman, Smith et al. 2012). As we have previously reported (Freeman, Smith et al. 2012), we used a tamoxifen-activated Cre recombinase (Cre^{ERT2}) targeted to the intestinal epithelium to achieve deletion of Smad4 expression in intestinal epithelial crypts of $Smad4^{lox/lox}$ mice. After tamoxifen treatment, we observed a chimeric depletion of Smad4 in our $K19Cre^{ERT2}$ mouse model with approximately 20% of colonic crypts showing loss of Smad4 expression one month post-tamoxifen treatment. We also crossed the $Lrig1Cre^{ERT2}$ mouse to a $Smad4^{lox/lox}$ background to generate mice with a greater percentage of intestinal crypts exhibiting knockout of Smad4 (Powell, Wang et al. 2012). By one month after Cre activation by tamoxifen treatment, we noted that approximately 80% of colonic crypts in the $Lrig1Cre^{ERT2}$ $Smad4^{lox/lox}$ crossbred mice no longer expressed Smad4, as determined by immunostaining (Figure 3.1). In Smad4-null crypts (denoted by black arrows, Figure 3.2B,E) we observed an increased proliferative zone by Ki67 immunoreactivity (Figure 3.2C,F). We observed that in the K19-driven Cre^{ERT2} mice that Smad4 wild-type crypts had 35.05 +/- 2.61% of cells positive for Ki67

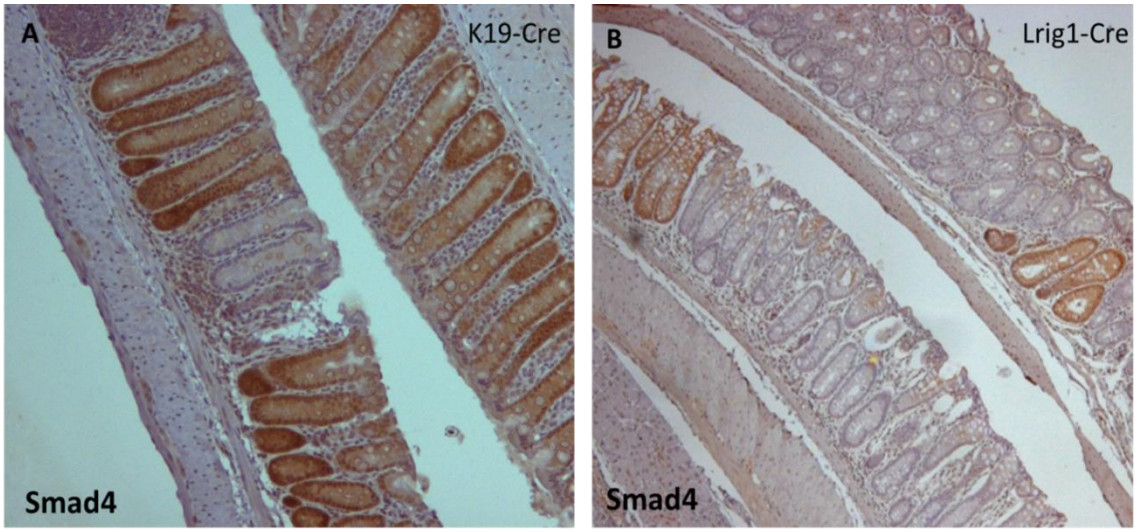


Figure 3.1. *Lrig1Cre^{ERT2}Smad4$^{lox/lox}$ mouse model has a higher percentage of recombined colonic crypts compared K19Cre^{ERT2}Smad4$^{lox/lox}$ via Smad4 immunohistochemistry*. (A,B) Smad4 immunostain on K19Cre^{ERT2}Smad4$^{lox/lox}$ and Lrig1Cre^{ERT2}Smad4$^{lox/lox}$, respectively, two weeks post tamoxifen treatment.

while Smad4 null crypts within the same section had 54.04 +/- 3.96% (p= 0.0286, Figure 3.2A). In our Lrig1-driven Cre model, Smad4 wild-type crypts had 39.62+/- 2.16% of Ki67 positive cells while Smad4 null crypts had 47.68+/- 1.7% (p= 0.0121, Figure 3.2D).

We assessed whether this extension in the proliferative zone resulted in the presence of fewer mature epithelial cells within the colonic crypt. We quantified enterocytes (Carbonic Anhydrase II positive cells per crypt) and goblet cells (Muc2 positive cells per crypt) in both the K19Cre^{ERT2}Smad4$^{lox/lox}$ and the Lrig1Cre^{ERT2}Smad4$^{lox/lox}$ models. We also quantified enteroendocrine (Chromogranin A positive) cell populations using high powered fields in the Lrig1Cre^{ERT2}Smad4$^{lox/lox}$ model that had sufficient frequency of Smad4-null crypts, due to the low number of enteroendocrine cells observed in individual crypts. In Smad4-null crypts (Figure 3.3 A,C) we observed a decreased number of mature enterocytes as determined by Carbonic Anhydrase II (Car II) immunostaining (Figure 3.3B,D). In the K19-driven Cre^{ERT2} mice, Smad4 wild-type crypts had 66.89 +/- 2.45% of cells positive for Car II while Smad4 null crypts had 52.11 +/- 3.15% (p= 0.0303, Figure 3.6A). In the Lrig1-driven Cre^{ERT2} mice, Smad4 wild-type crypts had 61.40 +/- 1.42% of cells positive for Car II while Smad4 null crypts had 43.49 +/- 1.31% ($p < 0.0001$, Figure 3.6B). With Muc2 staining, we noted that Smad4-depleted crypts, denoted by black arrows, had a decreased number of goblet cells (Figure 3.4). We observed in the K19-driven Cre^{ERT2} mice that Smad4 wild-type crypts had 12.44 +/- 2.47% of cells positive for Muc2 while Smad4 null crypts had 5.59 +/- 0.87% (p= 0.0313, Figure 3.6C). In the Lrig1Cre^{ERT2} mice, we noted that the goblet cell population percentage varied depending on region of the colon in which the Smad4 depleted crypts were found. The proximal colon has more goblet cells than the distal colon, so we separately analyzed the proximal and distal colonic regions (in the K19Cre^{ERT2} mice, most of the Smad4 depleted crypts were located in the distal colon). In proximal glands of the Lrig1-driven Cre^{ERT2} mice, Smad4 wild-type

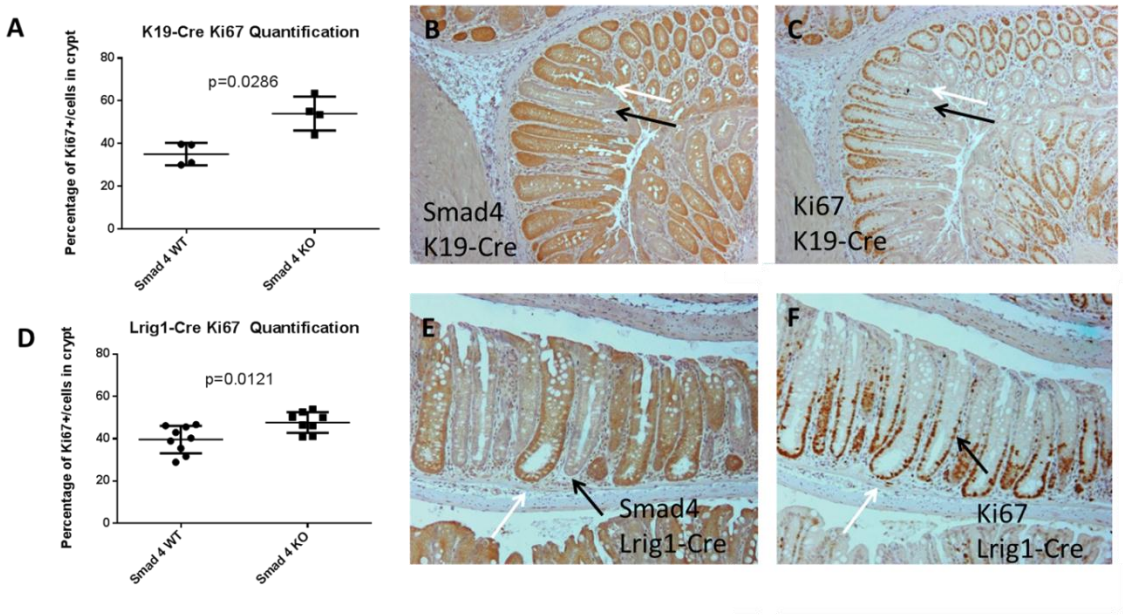


Figure 3.2. *Loss of Smad4 expression within the intestinal tract associates with increased proliferation in two mouse models.* (A) Quantification of Ki67 positive cells in the K19-Cre driven model comparing Smad4 wild-type (WT, n=4) and Smad4 null (KO, n=4) crypts (B) representative immunostain of Smad4 and (C) Ki67 one month post-tamoxifen treatment (D) Quantification of Ki67 positive cells in the Lrig1-Cre driven model comparing Smad4 WT (n=8) and Smad4 KO (n=8) crypts (E) representative immunostain of Smad4 and (F) Ki67 one month post-tamoxifen treatment.

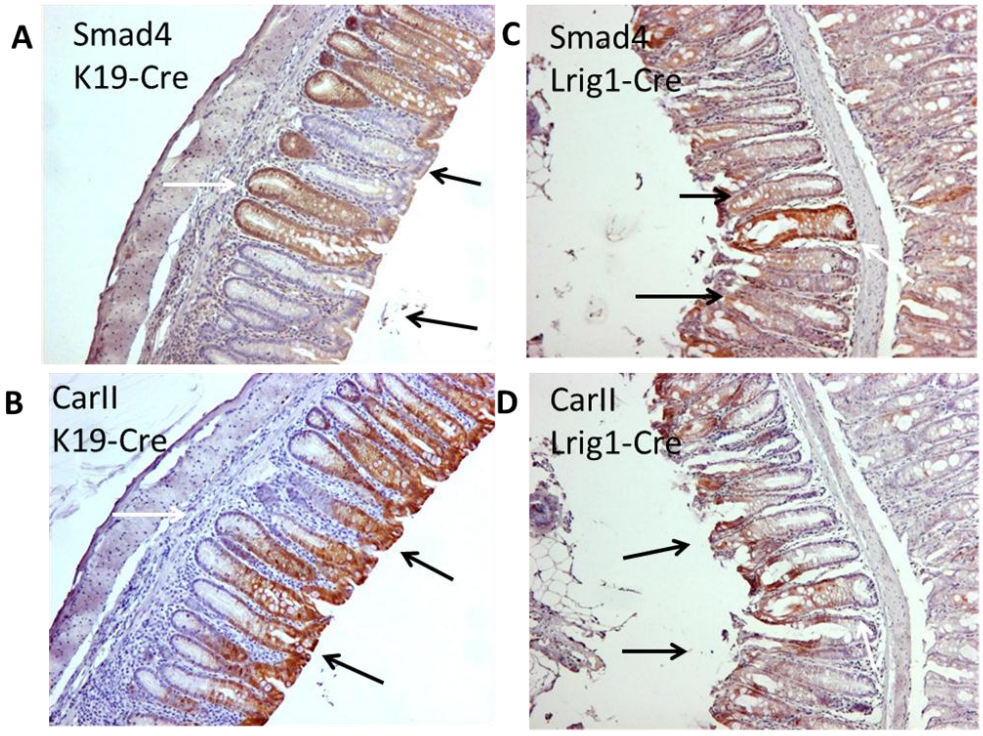


Figure 3.3. *Smad4 null crypts have fewer enterocytes.* Representative immunostain of Smad4 (A)and Car II (B) one month post-tamoxifen treatment of K19Cre^{ERT2}Smad4$^{lox/lox}$ mouse and representative immunostain of Smad4 (C) and Car II (D) one month post-tamoxifen treatment of Lrig1Cre^{ERT2}Smad4$^{lox/lox}$ mouse. Black arrow indicate Smad4 null crypts, white arrows indicate Smad4 expressing crypts.

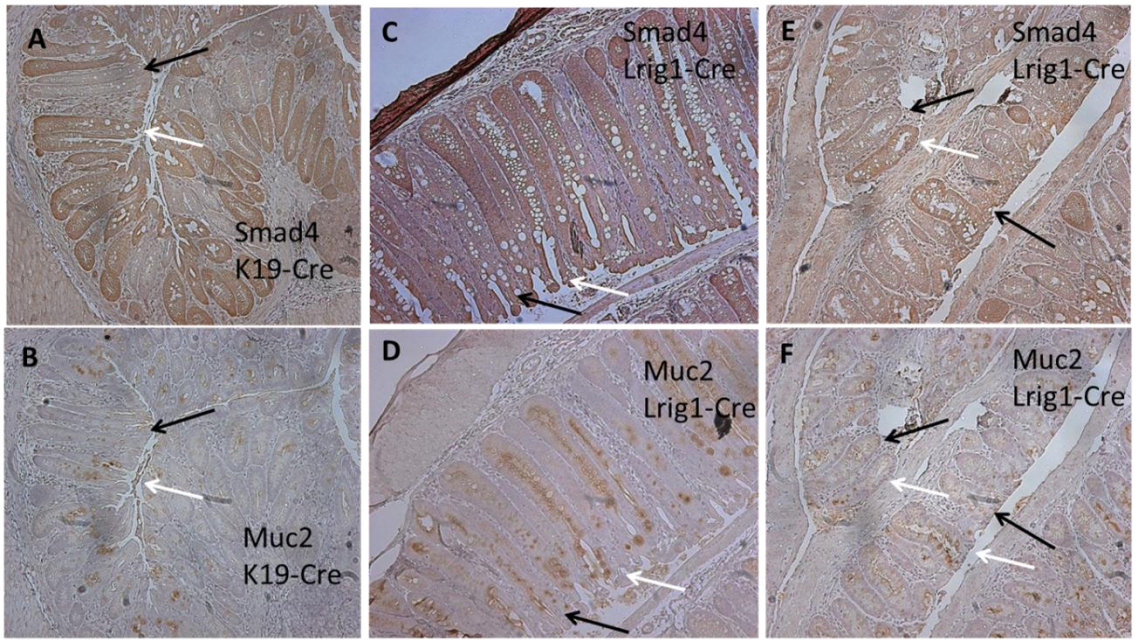


Figure 3.4. *Smad4 null crypts have fewer goblet cells.* Representative immunostain of Smad4 (A) and Muc2 (B) one month post-tamoxifen treatment of K19Cre^{ERT2}Smad4$^{lox/lox}$ mouse. Representative immunostain of Smad4 and of Muc2 in the proximal (C,D, respectively) and distal colon (E,F, respectively) one month post-tamoxifen treatment of Lrig1Cre^{ERT2}Smad4$^{lox/lox}$ mouse. Black arrow indicate Smad4 null crypts, white arrows indicate Smad4 expressing crypts.

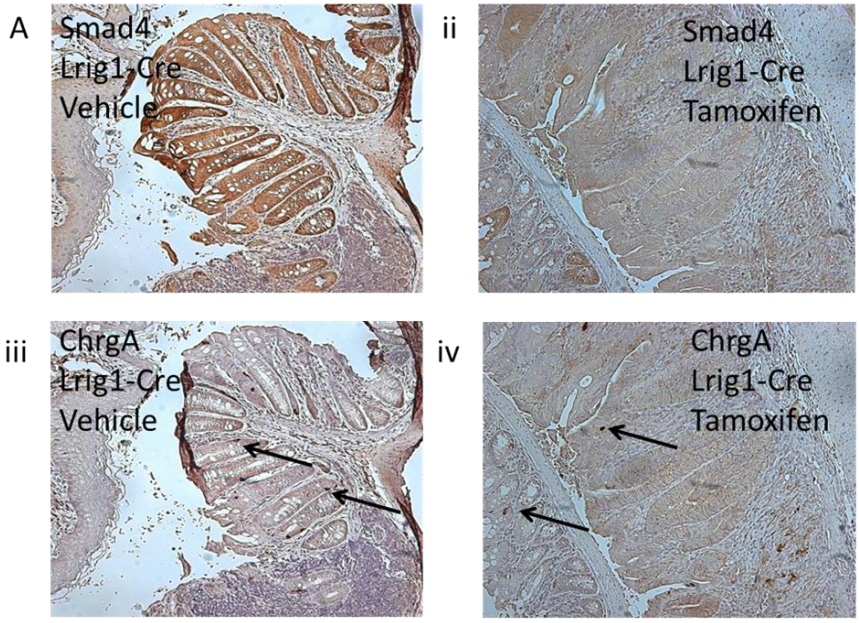


Figure 3.5. *Smad4 null crypts have fewer enteroendocrine cells.* Representative immunostain of Smad4 (A) and Chromagranin A (B) one month post-vehicle treatment of Lrig1Cre^{ERT2}Smad4$^{lox/lox}$ mouse and representative immunostain of Smad4 (C) and Chromagranin A (D) one month post-tamoxifen treatment of Lrig1Cre^{ERT2}Smad4$^{lox/lox}$ mouse. Black arrows represent positive staining for ChromograninA (B,D).

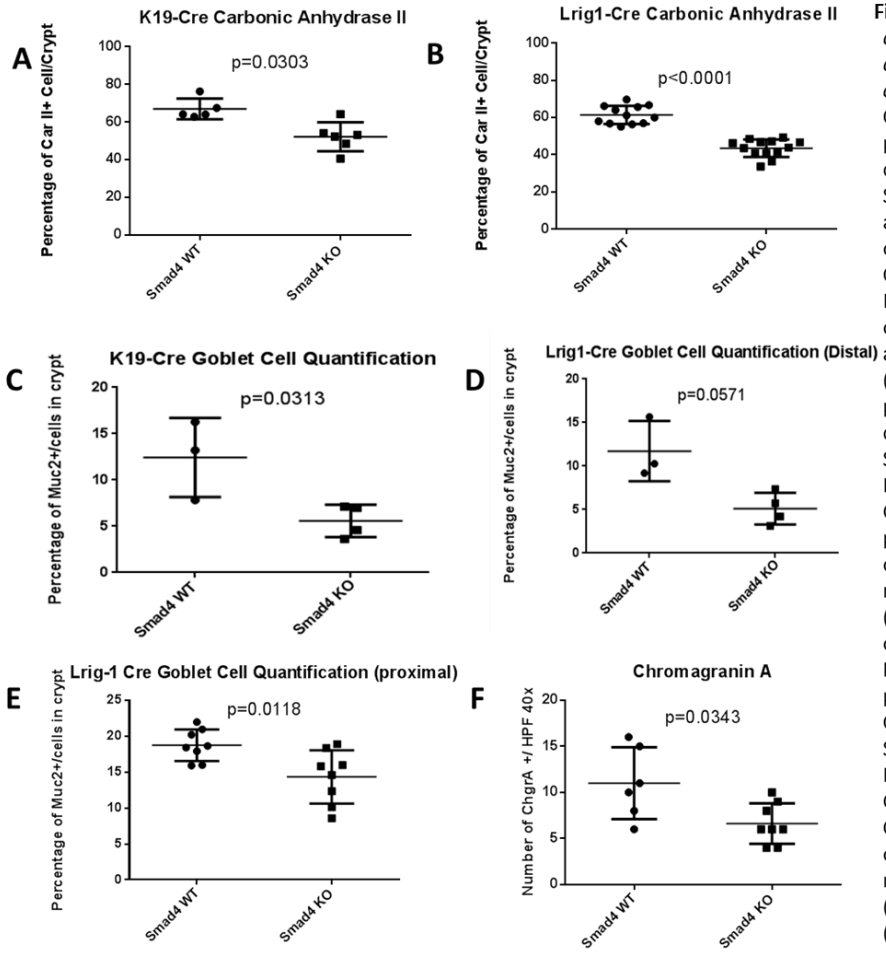


Figure 3.6. *Loss of Smad4 associates with decrease differentiation in the colonic crypt.* (A) Quantification of Carbonic Anhydrase II (Car II) positive cells in the K19-Cre driven model comparing Smad4 wild-type (WT, n=5) and Smad4 null (KO, n=6) crypts (B) Quantification of Car II positive cells in the Lrig1-Cre driven model comparing Smad4 WT (n=12) and Smad4 KO crypts (n=13) (C) Quantification of Muc2 positive cells in the K19-Cre driven model comparing Smad4 WT (n=3) and Smad4 KO (n=4) crypts (D) Quantification of Muc2 positive cells in the distal colon of the Lrig1-Cre driven model comparing Smad4 WT (n=8) and Smad4 KO (n=8) crypts (E) Quantification of Muc2 positive cells in the proximal colon of the Lrig1-Cre driven model comparing Smad4 WT (n=3) and Smad4 KO (n=4) crypts (F) (i) Quantification of Chromagranin A positive cells in the Lrig1-Cre driven model comparing Smad4 WT (n=6) and Smad4 KO areas (n=8).

crypts had 18.78 +/- 0.78% of cells positive for Muc2 while Smad4 null crypts had 14.36 +/- 1.32% (p = 0.0118, Figure 3.6E). In distal glands of the Lrig1-driven Cre^{ERT2} mice, Smad4 wild-type crypts had 11.71 +/- 2.00 % of cells positive for Muc2 while the Smad4 knockout crypts had 5.11 +/- 0.92% (p = 0.0571, Figure 3.6D). Using the $Lrig1Cre^{ERT2}$ mice for determining enteroendocrine population, we identified fewer enteroendocrine cells in Smad4 null areas with 6.625 +/- 0.78 Chromagranin A positive cell cells per high powered field compared to 11.00 +/- 1.60 Chromagranin A positive cell per high power field (Figure 3.5) for Smad4-expressing fields (p=0.0343, Figure 3.6F). These findings show that Smad4 null glands have an increased proliferative zone and have fewer mature differentiated cells than Smad4 wild-type glands, thus, suggesting that Smad4 signaling regulates the decision between proliferation and differentiation.

Smad4 Loss Results in Increased Intestinal Epithelial Permeability

Reduced intestinal epithelial cell maturation has been thought to lead to altered barrier function (Madara and Marcial 1984, Madara 1989). We therefore conducted studies to assess intestinal barrier function using low molecular weight FITC-dextran (3-5kDa), to test if loss of Smad4 increased intestinal permeability in tamoxifen treated $K19Cre^{ERT2}Smad4^{lox/lox}$ mice as compared to their control treated littermates with normal Smad4 expression. These experiments were conducted *ex vivo* with an Ussing chamber, in which FITC dextran was applied to the luminal aspect of distal colonic tissue, and samples were retrieved from the serosal aspect over time. In addition, we monitored transepithelial resistance to current in the same experimental system. We observed marked differences in the short circuit reading with greater resistance to electrical current in the colon from Smad4 wild-type (WT) mice as compared with Smad4-depleted colonic tissue (KO) (Figure 3.7A). In addition, we observed that over time, more FITC dextran permeated the colons with Smad4-null glands as compared with Smad4 WT colons (Figure 3.7B). We then performed *in vivo* FITC dextran

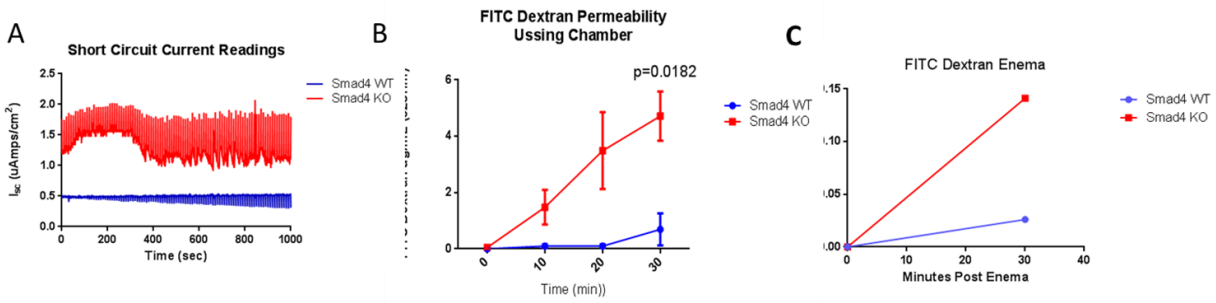


Figure 3.7. *Smad4 depletion leads to increased colonic permeability.* (A) Short circuit reads of 1mm^2 area of distal colons of Smad4 WT and Smad4 KO over time as indicated using a Ussing chamber(B) Measures of intestinal epithelial permeability to low molecular (5kD) weight FITC-Dextran over time (n=4 for Smad4 WT and Smad4 KO) (C) *In vivo* permeability in which Smad4 WT and Smad4 KO were given FITC-Dextran enemas. Peripheral blood was taken at 0min and 30min timepoints and serum was measured for fluorescence.

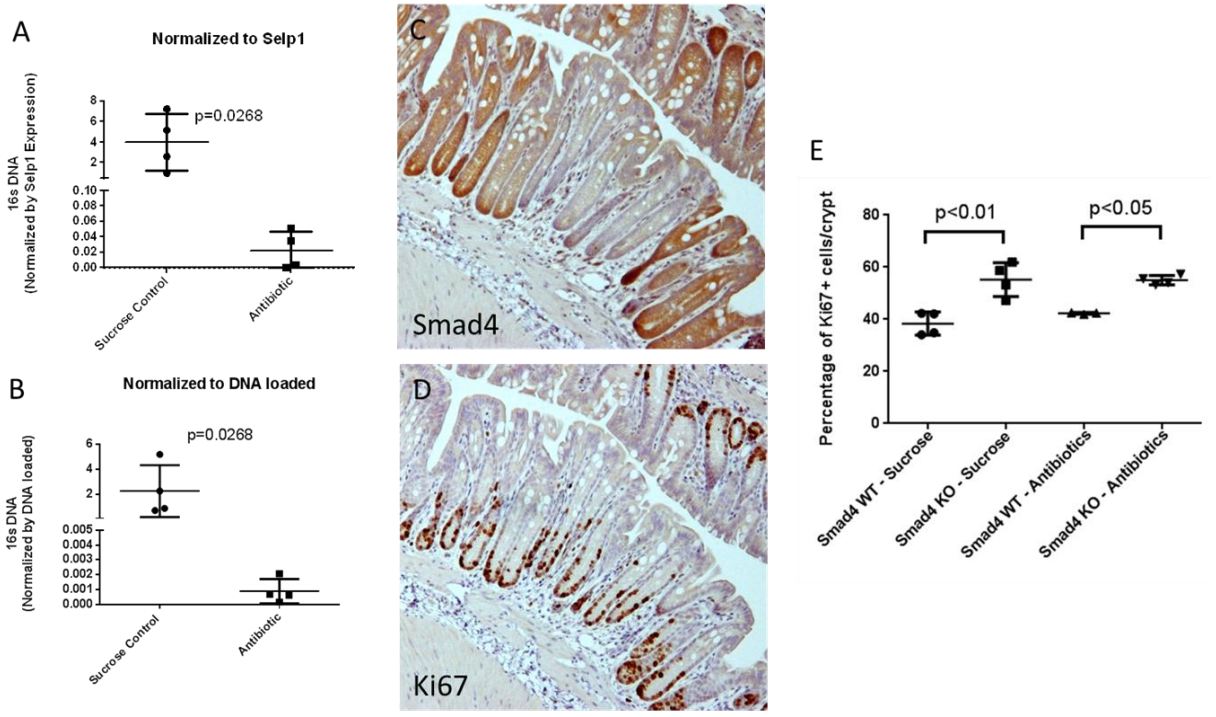


Figure 3.8. *Antibiotic treatment does not reverse the extension of proliferation observed in Smad4 null colonic crypts.* qPCR analysis of 16s DNA normalized to Selp1 (A) or normalized to DNA loaded (B) to examine bacterial load in fecal material harvested from mice either treated with sucrose control or antibiotic treatment. Representative immunostain of Smad4 (C) and Ki67 (D) two weeks post-antibiotic treatment. (E) Quantification of Ki67 positive cells in the Lrig1-Cre driven model comparing sucrose control WT (n=4) and Smad4 KO (n=4) crypts and antibiotic treated Smad4 WT (n=3) and Smad4 KO (n=4) crypts. Significance was determine by One-Way ANOVA with Bonferroni post-test to compare all columns.

permeability assays to confirm that our observations from the Ussing chamber experiments were not the result of sample preparation artifact. We observed a similar increase in FITC dextran in peripheral blood over a 30 minute period following FITC dextran delivery via enema (Figure 3.7C).

We then questioned whether the observed changes in proliferation could be explained due to increased injury due to microbiota being able to infiltrate the immature epithelial barrier (Buchon, Broderick et al. 2009, Liu, Lu et al. 2010). We treated vehicle and tamoxifen treated K19Cre^{ERT2}Smad4$^{lox/lox}$ mice with antibiotics for two weeks prior to sacrifice (one week post vehicle or tamoxifen treatment). To confirm the efficacy of our antibiotics treatment we performed qPCR analysis for bacterial 16s DNA and found that upon treatment with antibiotics that there was marked reduction in 16s burden whether normalized to Selp1 or to the amount of DNA loaded (Figure 3.8A, B) (Fu, Wei et al. 2011). We observed a similar extension of the proliferative zone in Smad4 null glands of antibiotic treated mice as in our sucrose-treated control mice supporting the hypothesis that the microbiota does not influence the proliferation phenotype (Figure 3.8C-E).

Smad4 Loss Is Not Associated with Up-regulation of Canonical Wnt Targets

Since we observed a decrease in both absorptive and secretory lineages and reported that Smad4 depletion results in increased levels of β-catenin mRNA and increased canonical Wnt signaling in tumor cells (Chapter II), we asked if the extension of the proliferative zone was associated with increased canonical Wnt pathway signaling. Ascl2 is a Wnt target gene that is transcriptionally activated by canonical Wnt signaling via β-catenin/TCF dependent activation (Jubb, Chalasani et al. 2006). We performed Ascl2 *in situ* hybridization, to assess alterations within the Wnt signaling pathway. Surprisingly, we found that in crypts in which Smad4 was no longer expressed that there was also reduced Ascl2 mRNA levels (Figure 3.9).

For more quantitative analysis, we isolated and cultured small intestinal enteroids from the K19Cre^{ERT2}Smad4$^{lox/lox}$ mice and induced recombination with hydroxy-tamoxifen *in vitro*. Treatment with hydroxy-tamoxifen induced Smad4 loss within the enteroids, as demonstrated at both the mRNA and protein levels (Figure 3.10A,B). We then analyzed the mRNA for differential expression of β-catenin and Wnt targets. Despite the increase in β-catenin mRNA, we were not able to detect increases β-catenin protein level (Figure 3.10B) or in mRNA for two canonical Wnt signaling targets (Axin2, c-Myc) (Figure 3.10 C-E).

Generation of Intestinal Smad4 Loss Gene Signature

Observing that our enteroid model did not implicate activation of the Wnt pathway with Smad4 loss, we conducted a global assessment of mRNA expression using RNA-Seq analysis to find out how Smad4 loss alters proliferation/differentiation independently of β-catenin. To this end, we utilized both the enteroids from our K19Cre^{ERT2}Smad4$^{lox/lox}$ mouse model and enriched isolated colonic epithelium cell fractions from the Lrig1Cre^{ERT2}Smad4$^{lox/lox}$ mice to generate an intestinal Smad4-depletion gene signature. RNA was isolated from Smad4 WT and KO enteroids for gene expression comparison. We treated Lrig1Cre^{ERT2}Smad4$^{lox/lox}$ with vehicle for control or induced recombination with tamoxifen treatment and waited two weeks prior to sacrificing the animals. Whole colons were harvested and epithelium was fractionated using an EDTA wash and mRNA prepared from the epithelial fraction. We first verified Smad4 depletion within the colonic epithelium (Figure 3.11A) and analyzed for differential expression for Wnt targets (Axin2, and Ascl2). We again observed increased expression in β-catenin at the mRNA level but no increase in downstream Wnt targets (Figure 3.11B-D).

We examined genes that exhibited Smad4-associated differential expression in the Smad4 knockout enteroids and colonic epithelium fractions from Smad4-depleted

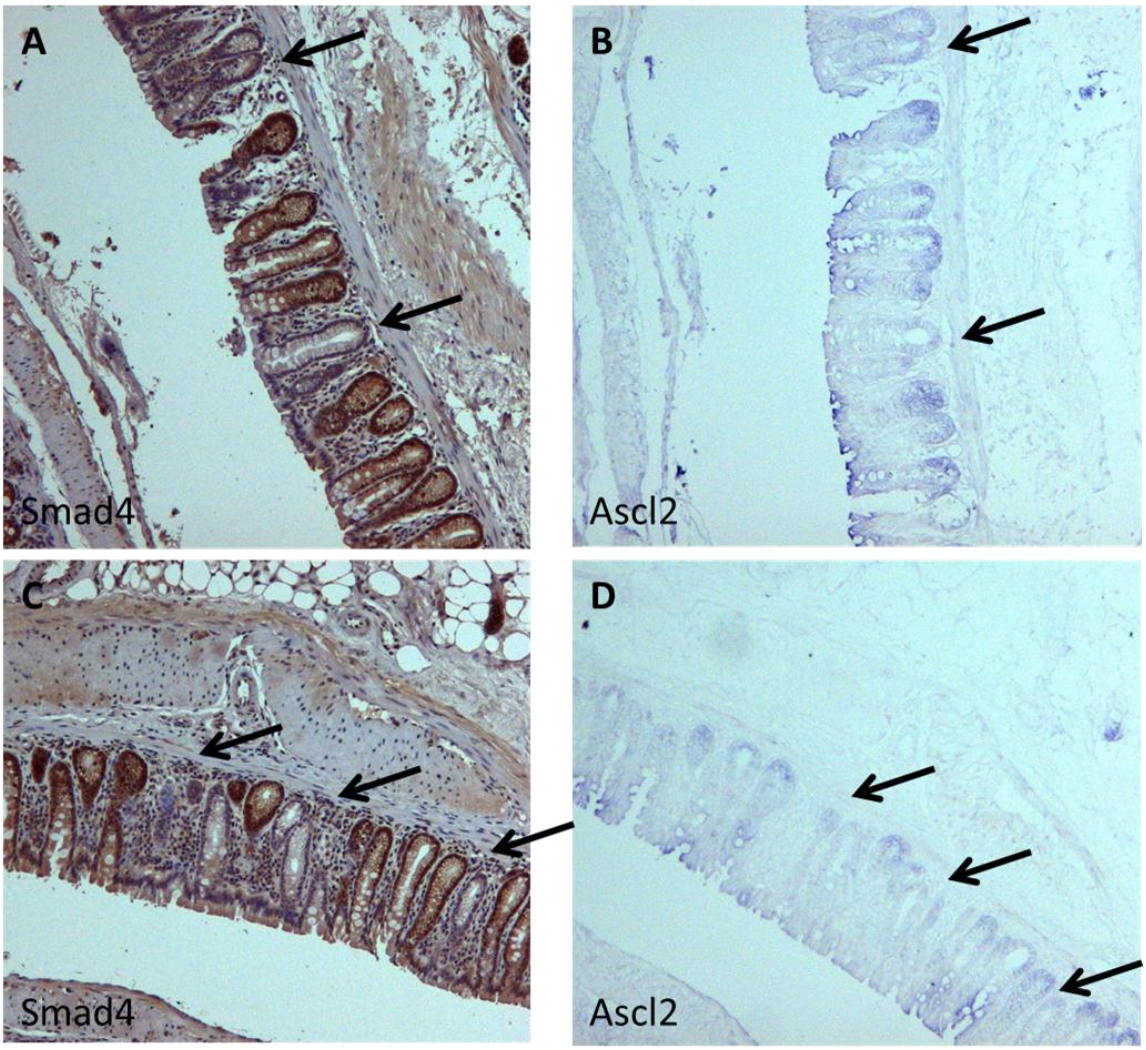


Figure 3.9. *Smad4 loss associates with loss of Ascl2 expression.* (A,C) Large intestine of K19-Cre^{ERT2} Smad4$^{lox/lox}$ mouse approximately one-month post tamoxifen treatment stained for Smad4 expression showing loss of Smad4 expression within the colonic crypts (B,D) Serial section of (A&C, respectively) showing in situ hybridization detecting Ascl2 expression. Black arrows indicate corresponding crypts from samples which are Smad4 null.

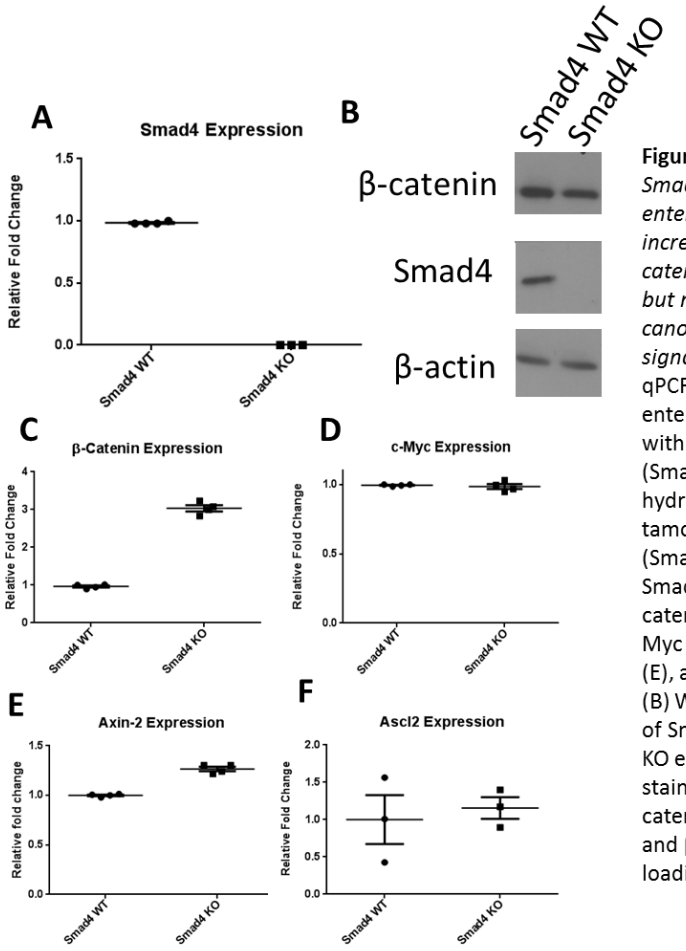


Figure 3.10. *Smad4 null enteroids have increased β-catenin mRNA but no increase in canonical Wnt signaling*. (A, C-F) qPCR analysis of enteroids treated with vehicle (Smad4 WT) or hydroxy-tamoxifen (Smad4 KO) for Smad4 (A), β-catenin (C), c-Myc (D), Axin-2 (E), and Ascl2 (F). (B) Western blot of Smad4 WT and KO enteroids stained for β-catenin, Smad4, and β-actin for loading control.

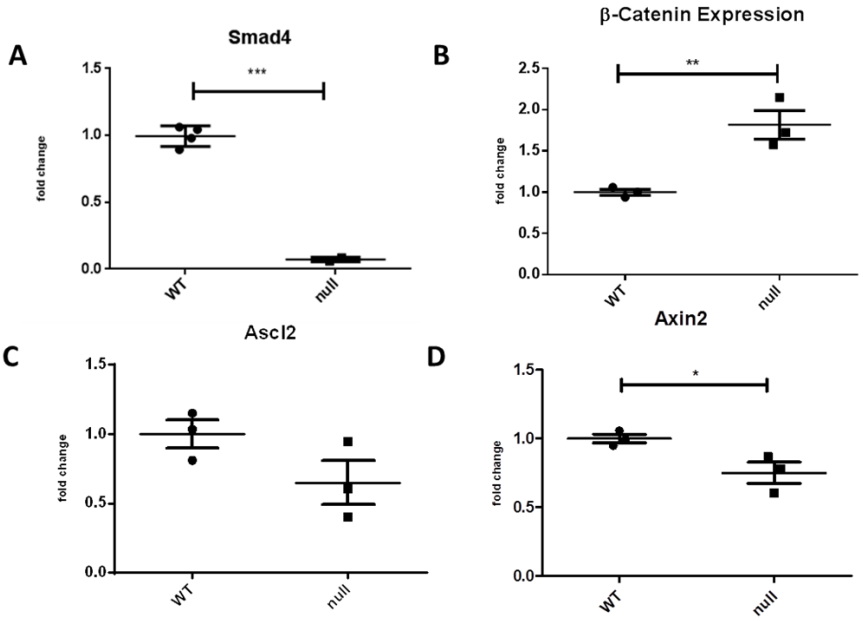


Figure 3.11. *Colonic Epithelium from Lrig1^{CreERT} shows no increase in Wnt Targets upon Smad4 depletion* (A-D) qPCR analysis of colonic epithelium treated with vehicle (Smad4 WT, n=4) or tamoxifen (Smad4 null, n=4) for expression of Smad4 (A), β-catenin (B), Ascl2 (C) and Axin-2 (D).

mice with adjusted p-value of less than 0.01 and identified 1550 genes altered in enteroids and 143 genes in colonic epithelium. We found 58 common genes between these two lists and filtered them to find genes that changed in the same direction both in the enteroid and colonic epithelium fraction yielding a 44 gene Smad4 associated signature. Ingenuity Pathway™ analysis (IPA) of the 44 gene signature (Figure 3.12, Table 2.1) revealed that the top clinical disorder associated with the signature was gastrointestinal disease (data not shown). We then performed network analysis with the up-regulated and down-regulated gene sets from the 44 gene signature. The down-regulated gene set implicated loss of TGF-β and BMP signaling with Smad4, their central mediator, thereby validating our model. The up-regulated genes showed Erk 1/2 as a central node implicating activation of this signaling cascade in Smad4 null tissues.

Erk Signaling is Activated in Smad4 Null Tissues

Based on the gene signature results, we then assessed phospho-Erk 1/2 (pErk1/2) levels in the enteroid culture system and found that in Smad4 depleted enteroids pErk1/2 levels were increased (Figure 3.13). To further assess the effect of Smad4 depletion on Erk1/2 activation, we examined pErk1/2 immunoreactivity in both K19Cre^{ERT2}Smad4$^{lox/lox}$ and Lrig1Cre^{ERT2}Smad4$^{lox/lox}$ mouse models to determine whether Erk signaling was specifically activated in Smad4 depleted colonic crypts. In both our K19 (Figure 3.13B,C) and Lrig1 (Figure 3.13D,E) models, we note that where Smad4 is depleted pErk 1/2 is detected at higher levels than surrounding Smad4- positive crypts suggesting that Smad4 depletion results in activation of Erk signaling in non-transformed intestinal epithelium. Previous studies have linked activation of Erk signaling with down regulation of Cdx2, a homeobox protein implicated in proper intestinal cell differentiation (Krueger, Madeja et al. 2009). We note that Smad4 null crypts in both our K19Cre^{ERT2} (Figure 3.14A,B) and Lrig1Cre^{ERT2} (Figure 3.14C,D) have lower expression of Cdx2.

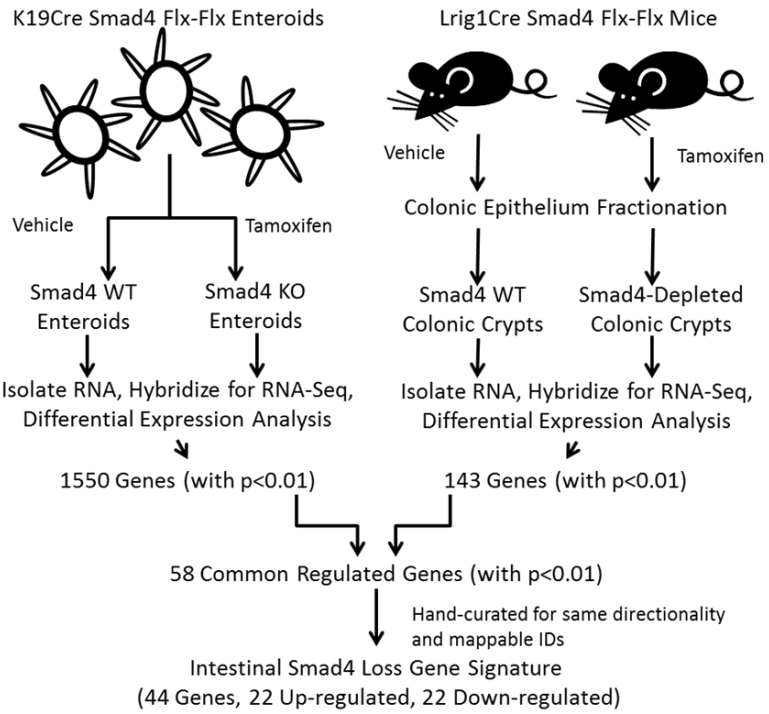


Figure 3.12. *Diagram of Workflow in Generating Intestinal Smad4 Loss Gene Signature.* RNA was harvested from Smad4 wild-type and Smad4 null tissues and hybridized for RNASeq. Differential expression analys was performed to determine what genes are differentially expressed in presence/absence of Smad4 expression. A gene list was generated for Ingenuity Pathway analysis.

Up-regulated Genes	Down-regulated genes
0610005C13Rik	6330403L08Rik
AL645846.1	Abcc4
Acer1	Anxa1
Ces1e	Bmp3
Cth	Bpgm
Gm1123	Col4a1
Gm4902	Cxcl5
Id1	Fam20c
Id3	Gna14
Me2	Il34
Oat	Jun
Ociad2	Lbp
Ostb	Mpp3
Ptges2	Mrc1
Retsat	Nrp1
Rhbg	Pamr1
Selenbp1	Pdgfa
Selenbp2	Pmepa1
Smad4	Rbpms
Smad6	Srgap1
Tbx3	Stxbp1
Ugt2b34	Tnip3

Table 3.1. *Genes comprising the Intestinal Smad4 Loss Signature* . List derived from differential expression in Smad4 null tissues from Enteroids and colonic epithelium isolated from Lrig1Cre^{ERT} mouse model.

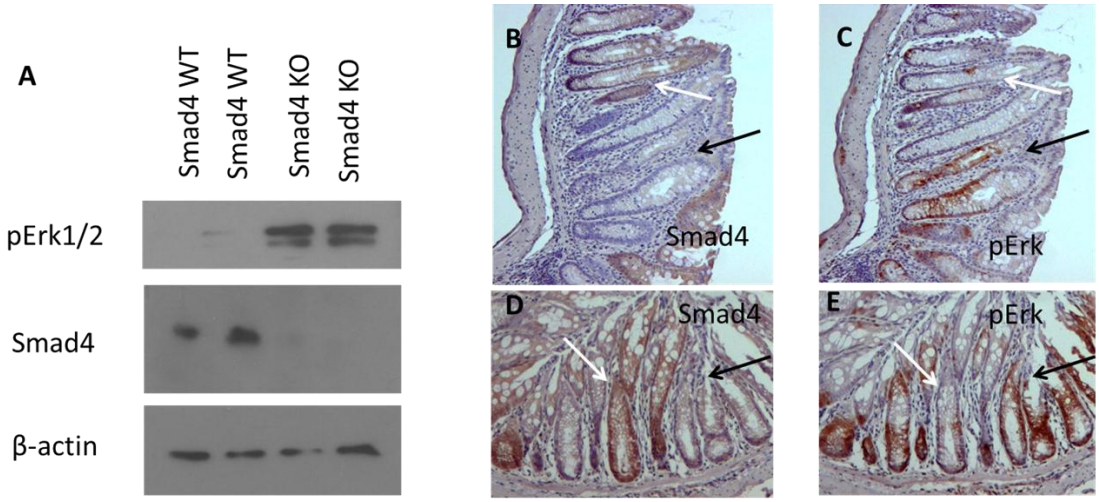


Figure 3.13. *Identification of Erk activation in Smad4 deplete tissues.* (A) Western blot analysis of Smad4 wildtype (WT) and Smad4 knockout (KO) enteroids for pErk, Smad4 and β-actin for loading control. (B-E) Representative immunostain of Smad4 (B,D) and pErk (C,E) one month post-tamoxifen treatment. (B,C) are serial sections from K19-Cre driven model; (D,E) are serials sections Lrig-1 Cre driven model. Areas of Smad4 loss is identified by black arrows, white arrows mark Smad4 WT crypts.

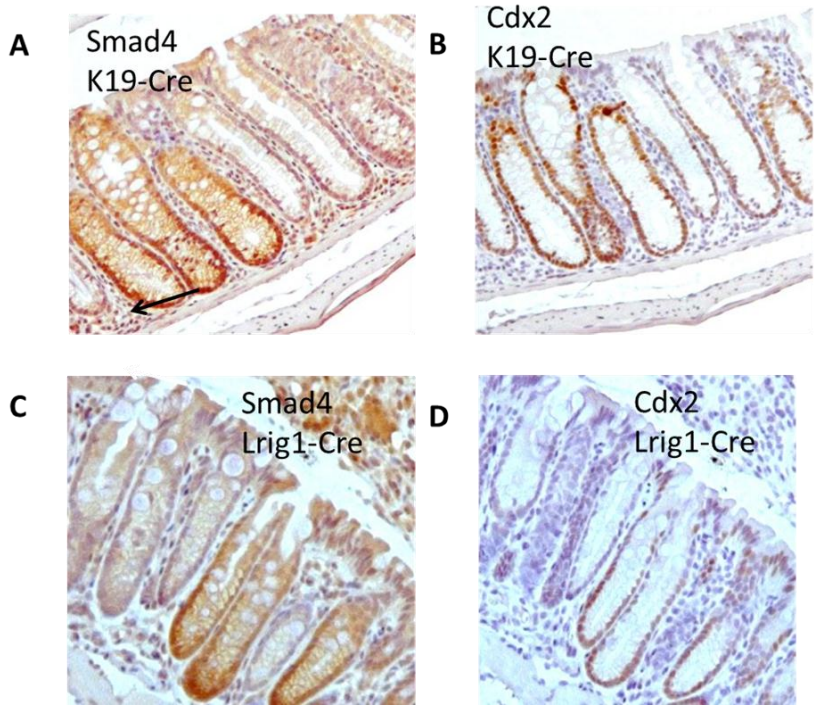


Figure 3.14. *Smad4 null crypts have reduced expresion of Cdx2.* Representative immunostain of Smad4 (A)and Cdx2(B) two weekspost-tamoxifen treatment of K19Cre^{ERT2}Smad4$^{lox/lox}$ mouse and representative immunostain of Smad4 (C) and Cdx2 (D) two week post-tamoxifen treatment of Lrig1Cre^{ERT2}Smad4$^{lox/lox}$ mouse. Black arrow indicate Smad4 null crypts, white arrows indicate Smad4 expressing crypts.

These findings could explain the decrease in differentiation we observe in Smad4 null crypts. Further studies focusing on the regulation of Cdx2 by Smad4 are needed to establish this as a mechanism by which Smad4 loss could alter intestinal homeostasis.

Intestinal Injury of Smad4 Deficient Mice Results in Development of Mucinous Adenocarcinoma

In the absence of other stimuli, the K19Cre^{ERT2} mice with depletion of Smad4 do not spontaneously develop intestinal tumors during a 1 year observation period. Since we observed a barrier defect in the Smad4-depleted mice, we reasoned that they might have an impaired response to mucosal injury. We used a well-characterized model of dextran sodium sulfate (DSS) in drinking water to observe how Smad4 loss would affect response to chronic colonic mucosal injury. To do this, we treated K19Cre^{ERT2}Smad4$^{lox/lox}$ with either vehicle or tamoxifen then waited one week before beginning three rounds 2.5% DSS treatment followed by recovery periods. We followed these mice monitoring weight and fecal content and observed that Smad4 knockout mice treated with chronic DSS developed rectal protrusion at three months of age. Upon sacrifice and dissection at three months of age, large non-polypoid, subluminal lesions were noted in the distal end of the large intestine of Smad4 knockout mice (n=7) while their control littermates (n=11) had no discernible phenotype (data not shown). Microscopic analysis showed that Smad4 knockout mice had developed invasive mucinous adenocarcinoma (Figure 3.15B) while their littermates showed no sign of injury (Figure 3.15A). Further magnification reveals dysplastic, mesenchymal-like cells with cyst structures forming along the basement membrane (Figure 3.15C,D). Mucin production in these lesions was confirmed with PAS staining (Figure 3.15E). Snail, a mesenchymal marker often indicative of epithelial to mesenchymal transition, was up-regulated in the cystic lesions (Figure 3.15F).

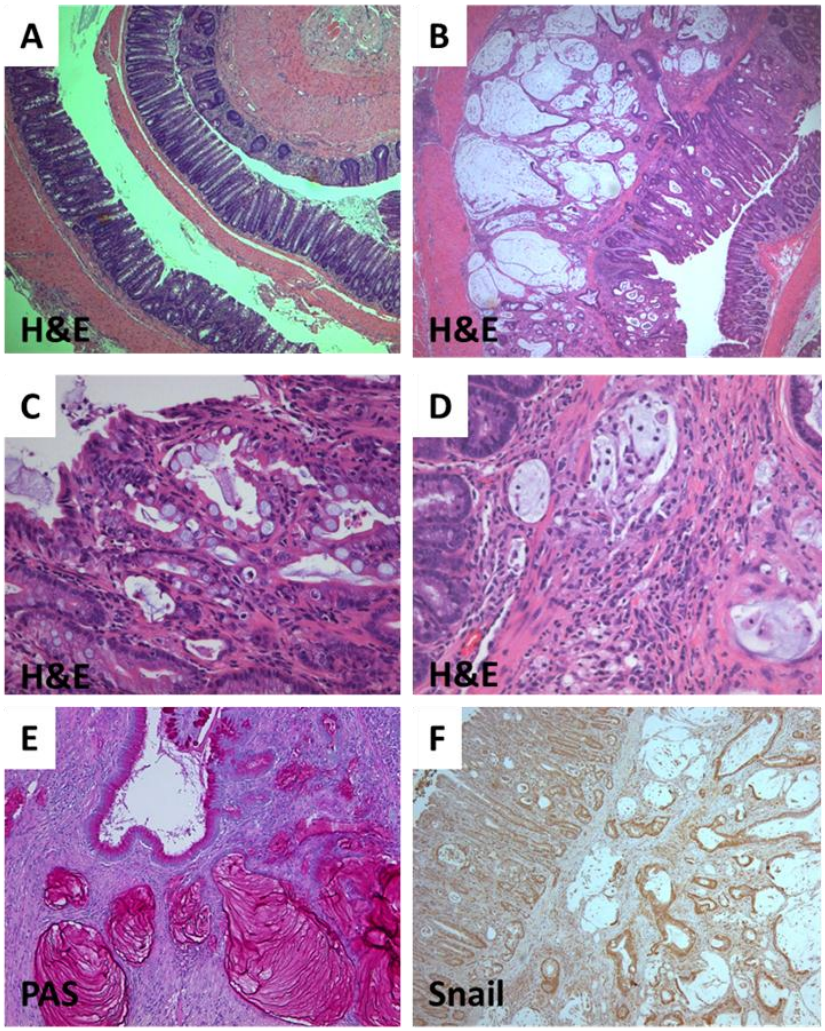


Figure 3.15. Smad4-deficient mice develop cystic adenocarcinoma post DSS treatment. Images of large intestine (A) Smad4$^{lox/lox}$ mouse and (B) K19-Cre^{ERT2} x Smad4$^{lox/lox}$ mouse (50x) approximately 100 days post-treatment with tamoxifen then three rounds of 2.5% DSS. (B) displays an area which has loss Smad4 expression via IHC analysis (data not shown). (C&D) Areas of (B) with cells undergoing squamous (C) and cystic (D) metaplasia (400x). Cystic regions are PAS positive (E) and have upregulation/nuclar localization of Snail (F) suggesting an alteration of differentiation along the colonic crypt axes.

Discussion

We noted in Chapter II that Smad4 null colonic crypts of K19Cre^{ERT2}Smad4$^{lox/lox}$ have increased BrdU incorporation and here have confirmed an increase in the proliferative zone by Ki67 staining, and we have also extended this observation to the Lrig1Cre^{ERT2}Smad4$^{lox/lox}$ model. We also observed that Smad4 null colonic crypts display a decreased population of differentiated colonocytes. We were unable to extend these observations to small intestine due to Smad4 expression being detected in >90% of the glands by immunohistochemistry (IHC) approximately one month post-tamoxifen treatment (data not shown). We postulate that the loss of Smad4 in the small intestinal crypt may lead to a competitive disadvantage resulting in repopulation of the small intestine by Smad4 expressing crypts over time, possibly through crypt fission. Future studies comparing the number of Smad4 null crypts over time will be needed to test this hypothesis. However, the recombination events in the large intestine for both the K19Cre^{ERT2}and Lrig1Cre^{ERT2} models have been maintained for greater than three months suggesting no competitive advantage of Smad4 expressing glands in colonic epithelium or that we have not observed the mice for enough time to observe the replacement of Smad4 null glands.

There are some discrepancies in numbers observed for different populations between our K19Cre^{ERT2} and Lrig1Cre^{ERT2} models (e.g. Car II stained 52.11 +/- 3.15% in K19-driven Cre^{ERT2} in Smad4 null crypts [Figure 3.6A] while Lrig1-driven Cre^{ERT2} mice, Smad4 null crypts had 43.49 +/- 1.31% cells stain positive for CarII). This may be attributed to the Cre^{ERT2} coding sequence being knocked-in to endogenous alleles, effectively deleting one copy of *Krt19* or *Lrig1*, respectively. These loci could have important roles in normal crypt development especially for the Lrig1Cre^{ERT2} model because the homozygous Lrig1Cre^{ERT2} mouse, with both alleles for *Lrig1* replaced with

Cre^{ERT2}, develop spontaneous duodenal polyps (Powell, Wang et al. 2012). Lrig1 functions as pan-ErbB inhibitor; therefore, complete loss of Lrig1 expression should enhance EGFR signaling. As discussed in Chapter I, EGFR signaling functions in maintenance of the intestinal stem cell niche (Biteau and Jasper 2011), and EGFR pathway activation leads to progression through the cell cycle (Oda, Matsuoka et al. 2005). However, we maintained our Lrig1Cre^{ERT2} model as a hemizygous Cre^{ERT2}, and no phenotype was previously reported for mice with a single knock-in allele. Regardless, we observed similar significant differences in cell populations (enterocytes, goblet cells, and enteroendocrine cells) in our two different conditional knockout models with fewer mature cells observed in Smad4 null crypts compared to Smad4-expressing crypts. Because both models gave similar results, we are confident that the decreased differentiation observed is due to the loss of Smad4 expression rather than *Krt19* or *Lrig1* haploinsufficiency.

In Chapter II, we observed that when Smad4 was knocked down in HCT116 cells, which possess an activating mutation of β-catenin, that β-catenin levels increased at both mRNA and protein levels, resulting in activation of canonical Wnt signaling (activation of TOP-Flash reporter). However, in the K19Cre^{ERT2} enteroids and Lrig1Cre^{ERT2} colonic models presented here, we observed that despite increases in mRNA expression of β-catenin that there is not an up-regulation of conventional Wnt signaling targets (Axin2, Ascl2, c-Myc, etc.). This could be attributed to the presence of an intact β-catenin degradation complex and wild-type β-catenin enabling cells to maintain a steady level of β-catenin protein despite an increased mRNA expression level. In Figure 3.9, we noted that Smad4 null crypts had less expression of Ascl2 by *in situ* hybridization. This suggests that Smad4 loss is altering the intestinal stem cell niche. Within our enteroid culture we noted that there is increased budding in Smad4-null

enteroids as compared to Smad4-expressing enteroids (data not shown) suggesting that there may be increased stem-like cells within the Smad4 null tissues. Understanding the role of Smad4 in maintenance of the stem cell niche within non-transformed epithelium could be investigated using our mouse models.

Within the Smad4 null mouse colonic crypts, we noted that there is decreased expression of Cdx2. Cdx2 is a homeobox protein that has been implicated in intestinal cell differentiation and identity (Krueger, Madeja et al. 2009, Grainger, Savory et al. 2010). Cdx2 has been shown to be down-regulated in colon cancer and inversely correlated with lymph node metastasis (Bakaris, Cetinkaya et al. 2008).Knocking out *Cdx2* expression in *Apc* mutant mice resulted in increased intestinal tumor burden (Aoki, Kakizaki et al. 2011). In the Caco-2 cell line, a colon cancer cell line that undergoes differentiation, Cdx2 expression was shown to decrease Wnt pathway activation, possibly through increased expression of APC and Axin2 (Olsen, Coskun et al. 2013), and stimulate an epithelial phenotype through increased expression of E-Cadherin (Natoli, Christensen et al. 2013). Within intestinal epithelium, ablation of Cdx2 resulted in increased proliferation and inability of cells to terminally differentiate into intestinal epithelium (Gao, White et al. 2009). Interestingly, in colorectal cancer cases, Cdx2 is rarely mutated suggesting that its expression is determined by transcriptional regulation (Hinoi, Loda et al. 2003). Understanding the mechanisms by which Smad4 signaling influences Cdx2 expression in our model system is an important future direction for this study.

Previous work that reported knocking out the BMP receptor pathway of signaling within the intestinal tract showed some similarities and some important differences compared to our Smad4 knockout models. It should first be noted that Smad4 serves as the central universal transcriptional mediator for all canonical TGFβ pathway signaling while silencing subfamily receptors only inhibit that specific pathway. Our results pointed

to an increased proliferative zone with fewer mature colonocytes in Smad4 null crypts. In one prior study utilizing the Mx-1-Cre to knockout Bmpr1a, the authors showed an extended proliferative zone within dysplastic regions of the small intestine that have lost expression of Bmpr1a; however, their analysis was skewed by these regions being transformed (He, Zhang et al. 2004). In addition, their recombination event was driven by Mx-1-Cre, and Mx-1 is expressed in multiple organ systems and functions in the defense of viral infections (Kuhn, Schwenk et al. 1995). This limits the interpretation of results since multiple systems and particularly the inflammatory system, could also be targeted. This is worth noting due to other observations that loss of Smad4 signaling within the T-cell compartment can also lead to intestinal tumor development (Kim, Li et al. 2006). Another group utilized Villin-Cre to remove intestinal expression of Bmpr1a (Auclair, Benoit et al. 2007) and found an extension of the proliferative zone within the small intestine. They also noted a decreased number of enteroendocrine cells within the small intestine but did not observe any changes in goblet, paneth, or enterocyte population number. However, their analysis of enterocytes population numbers may be misleading as they utilized Fatty Acid Binding Protein and Sucrase-Isomaltase, the former of which appears to mark most intestinal epithelial cells and the latter which, from their staining, does not mark all mature enterocytes. Later in their work, they performed qPCR analysis of duodenal and jejunal tissues and noted down-regulation of mature markers for enterocytes (not significant), Paneth, enteroendocrine, and goblet (significant ($p<0.05$ for all three). Given our results in which we note increased Erk activation and decreased expression of Cdx2 within Smad4 null colonic crypts, it would be interesting to investigate whether these other mouse models show a similar expression pattern as to further understand how loss of Smad4 signaling results in altered intestinal homeostasis.

From our studies, we conclude that Smad4 loss in the context of normal, non-transformed intestinal epithelium results in loss of epithelial homeostasis. Though we

observed an increase in β-catenin mRNA levels, this is not associated with Wnt pathway activation but with activation of Erk signaling. Following colonic mucosal injury, we note that Smad4 deficient mice develop mucinous adenocarcinomas without the need for germline mutation of *APC* or addition of a mutagen. We postulate that dysregulation of Erk signaling may lead to the development of intestinal carcinoma. Understanding the mechanism of Erk pathway activation could prove fruitful in designing a therapy in tissues which have loss Smad4 signaling.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

Brief Review

The epithelium of the intestinal tract is highly dynamic and strikes a careful balance between proliferation to maintain an intact interface and stem cell niche and differentiation to perform the functions such as nutrient absorption and barrier function. Perturbations in this balance have been implicated in carcinogenesis and can lead to increased proliferation and decreased differentiation as seen in colon cancer. Normally, Wnt signaling maintains the intestinal stem cell compartment and proliferative zone (Willert, Brown et al. 2003) while TGF-β family of signaling is thought to drive differentiation (Kosinski, Li et al. 2007) (Figure 1.4). Inappropriate Wnt pathway activation occurs frequently in colorectal cancer cases. Often this activation is associated with mutations in *APC* resulting in the inability to degrade β-catenin, the central mediator of the Wnt pathway (Laurent-Puig, Beroud et al. 1998). However, analysis of human tumor tissue reveals that mutation in *APC* or *β-catenin* was not sufficient to develop cancerous lesions (Figure 1.6) or to observe maximal levels of canonical Wnt signaling, suggesting that signals from the microenvironment may also affect Wnt signaling levels (Fodde and Brabletz 2007).

Among the other pathways implicated in colorectal cancer, alterations in TGFβ superfamily signaling occur in some 50-75% of all colorectal cancer cases, and Smad4, the central mediator of TGFβ superfamily signaling, is down-regulated in >50% of stage III patients (Isaksson-Mettavainio, Palmqvist et al. 2006). This loss of Smad4/TGFβ

signaling either early or late in tumor progression is associated with poorer prognosis (Alazzouzi, Alhopuro et al. 2005, Mesker, Liefers et al. 2009).The exact mechanism of how Smad4 signaling regulates Wnt signaling has not been reported nor has Smad4's role in maintaining intestinal homeostasis. We utilized biological models to investigate the role of Smad4 in maintaining intestinal homeostasis and in tumor progression.

In Chapter II (Freeman, Smith et al. 2012)**:** We investigated the regulation of β-catenin by Smad4 and described a new role of the tumor suppressor Smad4 in the transcriptional repression of β-catenin. In the context of transformed tissue, we observed an increase of canonical Wnt signaling and a dramatic increase in tumor burden when the *Smad4* gene was deleted in an *Apc* mutant background. We have extended the findings reported by Takaku et al (Takaku, Oshima et al. 1998) which showed that combined germline heterozygous mutation of *Apc* and *Smad4* alleles accelerated tumor progression in comparison to mutation of *Apc* alone. We did so by generating an inducible, tissue selective knockout of Smad4 expression in the adult mouse, thereby producing a model which more closely recapitulates the pathogenesis of human colorectal cancer. 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* (Costantini, Jho et al. 2002) in Smad4-null lesions compared to Smad4-expressing tumors from control animals. This finding was corroborated by analysis of tissue microarrays from human colorectal cases and observing that cases which do not retain Smad4 expression showed increased levels of β-catenin, supporting our murine model as a means to study human colorectal cancer.

Our *in vitro* experimental results show that inhibition of BMP signaling or loss of Smad4 in colon cancer lines can similarly augment β-catenin levels through a transcriptional mechanism, thereby increasing Wnt signaling. This finding suggests that Smad4 regulates β-catenin downstream of BMP signaling rather than TGFβ signaling at

least in the cell lines tested. Interestingly, our findings are consistent with 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 (Li, Huang et al. 2011). 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 Chapter III, we observed novel functions for Smad4 in intestinal homeostasis. We observed that loss of Smad4 is associated with an increased proliferative zone and a decrease in mature colonocytes within the large intestinal crypt. While loss of Smad4 in non-transformed intestinal epithelium results in increased expression of β-catenin mRNA there was no detectable increase in β-catenin protein or a functional increase in Wnt pathway activation. Instead, in this context, we observed an increase in Erk activation. Constitutive Erk activation has been associated with inhibition of differentiation in association with down-regulation of Cdx2, a homeobox protein implicated in intestinal differentiation (Lemieux, Boucher et al. 2011). We observed that Smad4 null crypts also have lower expression of Cdx2 which could potentially explain our observation of decrease in mature colonocytes. DSS treatment of mice with Smad4 null crypts resulted in development of invasive mucinous adenocarcinoma suggesting that persistent Erk signaling could predispose this model to tumorigenesis. Overall, these results provide insights into the role of Smad4 in maintaining normal intestinal homeostasis and how its loss can promote tumorigenesis.

The contribution of this research has been to further elucidate how Smad4 signaling functions in normal intestinal homeostasis and that Smad4 signaling suppresses canonical Wnt signaling in the context of transformed tissue. This contribution is significant because a majority of all colorectal cancer cases have mutations in Smad4/TGFβ/BMP signaling (Isaksson-Mettavainio, Palmqvist et al. 2006).

Our work shows that loss of Smad4 in tumors could promote tumor maintenance/dissemination via cell autonomous effects such as proliferation. These results suggest that restoring Smad4 activity may make tumors less aggressive. While gene therapy remains non-viable for treating most human diseases, further research to replicate the effects of Smad4 through therapeutic treatment could prove fruitful. This therapeutic approach could be utilized in the >80% of colorectal cancer cases in which the Wnt pathway is aberrantly activated.

Future Direction for Smad4 Repression of β-catenin Transcription

We observed that both Noggin treatment and Smad4 depletion result in increased transcription of β-catenin (Figures 2.2, 2.4, 3.10, and 3.11). Further, we noted that this regulation of β-catenin is lost upon expressing β-catenin under the control of a heterologous promoter (Figure 2.3). There are at least 12 potential Smad binding elements in the 3400bp 5' to the *CTNNB1* (the gene encoding β-catenin) transcriptional start site along with several other consensus transcription factor binding sites (Figure 4.1A) suggesting that Smad4 may directly regulate the transcription of the *CTNNB1* gene. Using a *CTNNB1* promoter reporter plasmid assay, we noted that Smad4 depletion results in increased reporter activity (Figure 4.1B). This all supports the hypothesis that Smad4 has a transcriptional repression effect upon the *CTNNB1* gene; however, much remains to be elucidated concerning the mechanism of this transcriptional regulation.

First, what region of the *CTNNB1* promoter is needed for Smad4 signaling to repress expression? Using the promoter reporter construct described (Nollet, Berx et al. 1996), we can generate 700bp fragment deletions from the 5' end to identify regions that are important for regulation by Smad4 following treatment with BMP and/or Noggin and with and without Smad4 depletion by siRNA. Based on the deletion studies, we could pinpoint fragments of interest in the promoter to generate a series of mutant constructs,

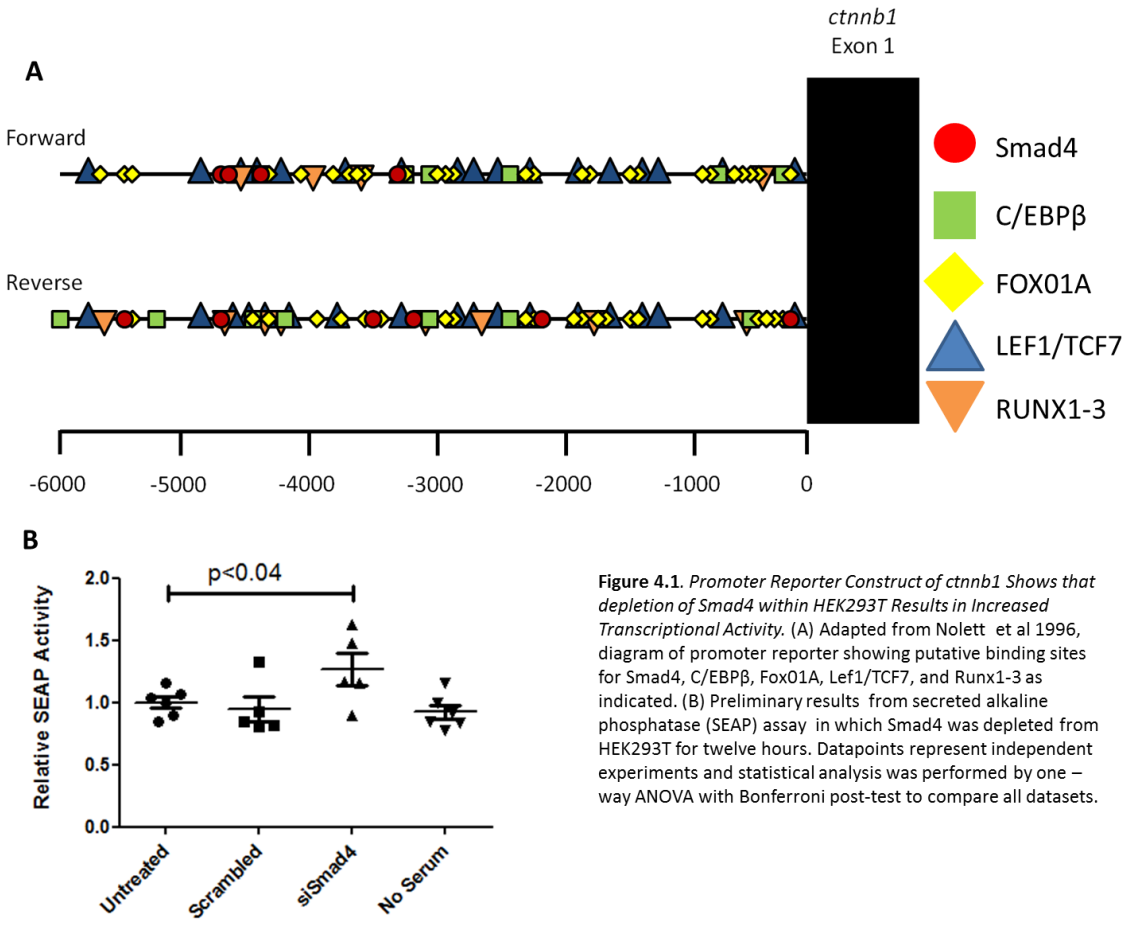


Figure 4.1. *Promoter Reporter Construct of ctnnb1 Shows that depletion of Smad4 within HEK293T Results in Increased Transcriptional Activity.* (A) Adapted from Nolett et al 1996, diagram of promoter reporter showing putative binding sites for Smad4, C/EBPβ, Fox01A, Lef1/TCF7, and Runx1-3 as indicated. (B) Preliminary results from secreted alkaline phosphatase (SEAP) assay in which Smad4 was depleted from HEK293T for twelve hours. Datapoints represent independent experiments and statistical analysis was performed by one – way ANOVA with Bonferroni post-test to compare all datasets.

also fused to a reporter construct, and assess the activity of these reporters under identical treatment conditions. This will determine which specific sequence elements are required for the suppressive effect of Smad4 upon β-catenin promoter driven reporter expression.

Second, is the transcriptional regulation a result of direct binding of Smad4 to the *CTNNB1* promoter? Chromosome immunoprecipitation can be performed on prepared nuclear lysates using antibodies against Smad4 and PCR amplification of Smad4-responsive elements described above. Quantitative PCR amplification of putative Smad binding sites from the *CTNNB1* promoter regions can be compared to binding sites of known BMP targets (e.g. Id2). Promoter binding will then be corroborated by electrophoretic mobility shift assays. In order to further determine the co-regulators that may be cooperatively binding with Smad4 present, we can take a candidate approach with a series of co-immunoprecipitation (Co-IP) experiments to determine whether immunoprecipitated nuclear Smad4 is bound to Smads1/5/8, Smads2/3 or with the co-repressors: E2F4/5, Nkx3.2, Runx2, p107, Sin3, or HDAC4 in nuclear lysates(Figure 4A). Once candidate co-repressors are identified, siRNA knockdown of each candidate co-repressor can be performed to elucidate which of the co-repressors are necessary for BMP-mediated transcriptional repression of the *CTNNB1* gene.

We predict that reporter activity will increase when the BMP responsive elements of the β-catenin promoter are mutated since Smad4 would no longer be able to bind to the promoter region. We have experimentally demonstrated the repressive effect of BMP-mediated Smad4-dependent signaling on β-catenin mRNA expression and expect to show that Smad4 regulates transcription at the β-catenin promoter. Our experiments are designed to determine whether these effects are direct or indirect. If there is no change in reporter activity after mutating single regions of interest, combination of mutation sites encompassing possible response elements will be tested. We anticipate

that mutation of specific gene elements will result in some change in the affinity of co-regulators, such as HDACs to the *CTNNB1* promoter; detection of these interactions may require chemical cross-linking to capture transient complex formation on the promoter (Massague, Seoane et al. 2005). In addition, tandem mass spectroscopy studies can increase the sensitivity and may be able to identify unanticipated, difficult to detect, and possibly novel, binding partners present in the repression complex.

Finally, we have shown an increase in β-catenin mRNA expression upon Smad4 depletion in the presence of an intact β-catenin degradation complex does not translate into increased β-catenin protein levels/increased Wnt pathway activation. We hypothesize that the APC-degradation complex maintains a constant level of β-catenin protein even when β-catenin mRNA is elevated and that Smad4 loss only alters Wnt pathway activation when the APC-degradation complex is inhibited or down-regulated. However, we have yet to test this experimentally. We are currently breeding $APC^{\Delta 1638/+}Lrig1Cre^{ERT}Smad4^{lox/lox}$ mice in an attempt to answer this question by isolating mRNA and protein from colonic epithelium of these mice four days post treatment with either vehicle or tamoxifen. With this loss/truncation of a single allele of APC, we would be able to determine if loss of Smad4 can increase β-catenin protein level and increase in Wnt targets (such as Axin2, c-Myc, and Ascl2) when there is reduction, rather than complete loss of the degradation complex. This may uncover a physiological as opposed to pathophysiological role for Smad4. Given that $APC^{\Delta 1638}$ model depends on loss of heterozygosity to produce intestinal tumors, the single copy of wild-type *APC* may be enough to prevent β-catenin protein accumulation and activation of Wnt signaling. However, we predict that upon depletion of Smad4 that in the presence of one mutant copy of APC that we should see elevated levels of β-catenin protein and expression of Wnt targets.

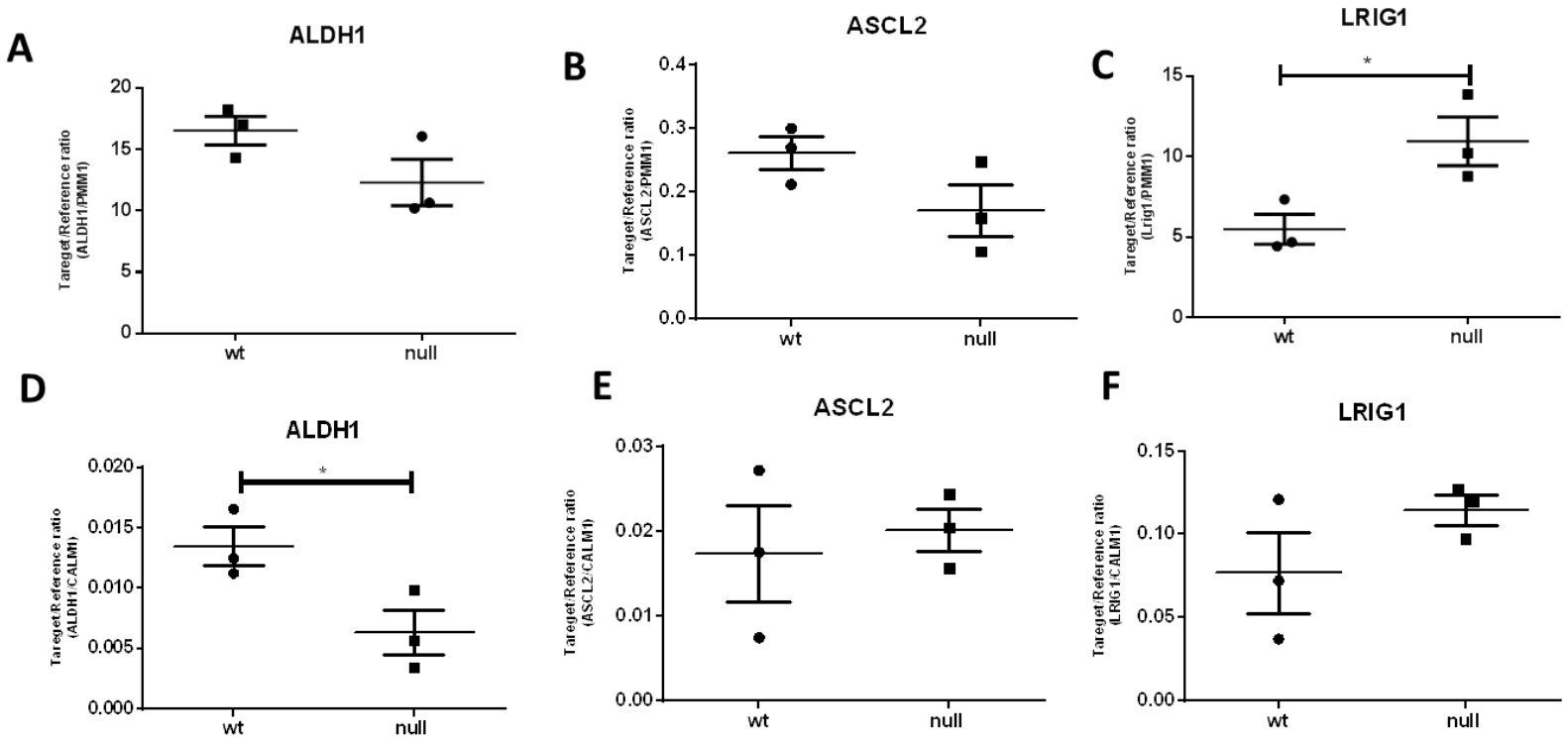


Figure 4.2 *Loss of Epithelial Smad4 Alters Expression of Intestinal Stem Cell Markers* . qPCR analysis of (A-C) epithelium isolated from Lrig1Cre^{ERT} Smad4$^{lox/lox}$ mice one week post vehicle or tamoxifen treatment and of (D-F) of enteroids with and without Smad4 expression. Analysis of ALDH1 (A,D) ASCL2(B,E) and LRIG1 (C,F) with significance determine by student's t-test. Specimens were generated by Anna Means, Ph.D. and Daniel Sharbel while analysis was performed by Connie Weaver.

Future Direction for Smad4 Signaling in Maintaining Normal Intestinal Homeostasis

We have presented data in Chapter III (Figure 3.9) that show that crypts which are null for Smad4 expression have qualitatively less Ascl2, a canonical Wnt pathway target gene, mRNA by in situ hybridization. On further investigation of this observation in the enteroid model and in isolated colonic epithelium from our Lrig1Cre^{ERT} Smad4$^{lox/lox}$ we see no significant difference in Ascl2 expression (Figure 4.2 B, E). However, we did observe that certain markers such as Lrig1 (Figure 4.2 C) in our colonic epithelium are increased in its expression. This suggests that loss of Smad4 expression results in alterations within the intestinal stem cell niche. Within the intestinal tract, injury results in increased Lrig1 expressing cells within the crypt (Powell, Wang et al. 2012). This could suggest that we are altering the local environment to an injured state (discussed in detail below). We are currently generating mice for frozen sections for immunofluorescence to investigate if this increased expression of Lrig1 mRNA results from a greater number of cells expressing Lrig1 or from the same number of cells expressing higher levels of Lrig1.

We observed in Chapter III that Smad4 depletion followed by treatment with DSS results in development of invasive mucinous adenocarcinoma without the presence of a germline mutation in APC (Figure 3.15). While we have observed an increase in Erk pathway activation with Smad4 loss, the manner of tumorigenesis remains to be elucidated. To accomplish this, we are collecting mRNA from our tumor and normal adjacent tissue to identify what pathways are activated/have mutations in them allowing us to understand what is driving/sustaining the tumor. We are also harvesting large intestine at earlier time-points to capture what occurs early in this adenocarcinoma pathogenesis. Based on the mucinous nature of these lesions, we hypothesize that an activation of PI3K pathway may be implicated in tumor development; however, this is

based solely on the phenotype of the tumors (Leystra, Deming et al. 2012).

In Chapter III, we observed activation of Erk signaling was associated with loss of Smad4. Previous work has pointed to activation of Erk signaling by BMP/TGF pathway signaling (Kleeff, Maruyama et al. 1999, Zhang 2009). More pertinently, prior work in Capan-1 cells, a Smad4-mutant pancreatic adenocarcinoma, showed that treatment with BMP2 resulted in activation of the MAPK pathway and that re-expression of Smad4 resulted in loss of the MAPK pathway activation by BMP2 treatment (Kleeff, Maruyama et al. 1999). This result suggesting that BMP treatment resulted in non-canonical activation of the MAPK pathway prompted us to examine whether the activation of Erk that we observed in our model systems could be attributed to non-canonical BMP signaling. To test this, we treated Smad4 null colonocytes and Smad4-depleted (via siRNA) HEK293T cells with BMP2 or Noggin. We observed that no change in pErk levels in Smad4-expressing cells (Figure 4.3 A,B). However, Noggin treatment of Smad4 null colonocytes or Smad4-depleted HEK293T cells resulted in lower pErk levels suggesting that BMP signaling could be activating Erk signaling through a non-canonical pathway. Interestingly, we also observed that Smad4 null/depleted cells actually displayed lower levels of pErk as compared to Smad4 expressing cells, which is opposite of what we observed in our enteroid system and within colonic epithelium (Figure 3.13). The reasons for these paradoxical observations are not entirely clear; however, we postulate that these differences may be due to different response in 2D monolayer cell cultures and the enteroids/crypts that represent a 3D system in which differentiation is able to transpire. Further studies are needed to further interrogate the non-canonical signaling downstream of the TGF-β/BMP receptor activation. These experiments will include comparative treatments with Noggin or exogenous BMP to determine if BMP treatment can specifically cause activation of Erk in this system. It will also be important to extend this analysis to a cell line such as Caco-2 that has been

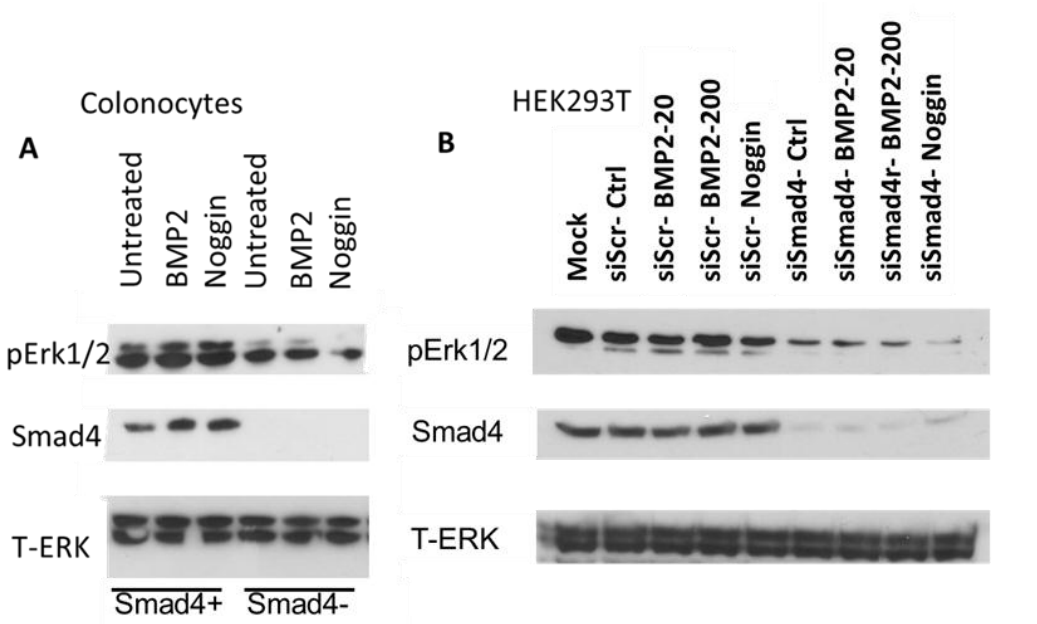


Figure 4.3. *Noggin treatment in Smad4-depleted tissues results in decreased Erk Signaling.* Representative (n=3) immunoblots of (A) colonocytes with and without Smad4 and (B) HEK293T that were siRNA-treated with scrambled or targeted Smad4 siRNA prior to being treated treated with BMP2 or Noggin as indicated for 3hrs then lysed to examine expression of Smad4, pErk and total Erk (T-ERK). Numbers above lanes (e.g. 20, 200) represent amount of reagent added to cell culture in ng/mL. All treatments were performed for three hours prior to lysis.

shown to differentiate in culture.

The focus of this project has been primarily upon how loss of Smad4 within the epithelial compartment affects intestinal homeostasis by measuring alterations within the epithelium itself; however, the intestinal tract is composed of more than simply epithelium. While this gross simplification enabled us to focus on specific questions in regards to epithelial biology, there is data to suggest that we are altering the surrounding mesenchyme within the tissue in a cell non-autonomous manner. We have begun investigating these alterations using our Lrig1Cre^{ERT}Smad4$^{lox/lox}$ mouse model. After induction of recombination with tamoxifen treatment, we harvested the colon and have isolated the colonic epithelium using an EDTA wash and what remains is our mesenchymal fraction. We have submitted these samples for RNA-Seq and found that we have enrichment for B-cell signaling within the colonic mesenchyme of Lrig1Cre^{ERT}Smad4$^{lox/lox}$ treated with tamoxifen versus those mice that have been treated with vehicle (data not shown). We also have generated evidence from whole tissue isolation that loss of Smad4 within the epithelial compartment can influence inflammatory aspects of intestinal tissue. We found that both T-cells (Figure 4.4A,B) and macrophages (Figure 4.4D-G) were surrounding Smad4 null crypts. Using the Luminex assay (Barry, Asim et al. 2011) and whole tissue lysate from colonic epithelium, we observed an increased expression of MIP3a (Ccl20) (Figure 4.4C). We are planning to flow sort immune cells in our Lrig1Cre^{ERT} Smad4$^{lox/lox}$ model to ascertain shifts which occur in inflammatory cells due to loss of Smad4 expression within the epithelial compartment. From our limited data, we would anticipate that an overall increase in the number of tissue T-cells and macrophages within colonic tissue; however, we are unsure whether these infiltrating immune cells will be immunosuppressive or pro-inflammatory.

Further studies were performed to ascertain whether Ccl20 expression could be originating from intestinal epithelium. We examined Ccl20 in the colonic epithelial

fraction from our Lrig1Cre^{ERT} Smad4$^{lox/lox}$ mouse model and found that Smad4 null cells expressed higher levels of CCL20 (Figure 4.5C). We utilized immortalized colonocytes isolated from Smad4$^{lox/lox}$ mice and treated them with either Adeno-GFP for control or Adeno-Cre to induce recombination. We then clonally selected cells for loss of Smad4 expression (Figure 4.5A). We examined these cells for differential expression of CCL20 and found that Smad4 null colonocytes expressed more Ccl20 mRNA than did Smad-4-positive colonocytes (Figure 4.5B). We then asked if this regulation could perhaps be regulated by BMP signaling or is mediated through secreted stromal ligands. To this end, Smad4 wild-type (wt) colonocytes were treated with BMP2 and/or conditioned media from immortalized mouse pericryptal fibroblasts (IMPF). In control (fresh) media, we found that with increasing exogenous BMP2 there was a slight decrease in Ccl20 expression (Figure 4.5D). Since BMP inhibitors are expressed by stromal cells surrounding intestinal epithelium, we tested the hypothesis that stroma normal regulates Ccl20 via the BMP pathway. There was a significant increase noted in Ccl20 expression when Smad4 wt colonocytes were treated with conditioned media from IMPF suggesting that there may be cross-regulation of epithelial expression of Ccl20 through surrounding mesenchyme. Adding increasing amounts of exogenous BMP2 abrogated this increase in Ccl20 expression, suggesting that pericryptal fibroblasts regulate Ccl20 expression via repression of BMP signaling. This repression becomes constitutive when Smad4 is deleted.

We hypothesize that IMPFs secrete a BMP pathway antagonist (e.g. Noggin, Gremlin, etc.) to counteract the regulation of BMP signaling upon CCL20 expression. We could knock down candidate factors from IMPF and observe which factor is necessary for this regulation of CCL20. We ultimately plan to confirm these findings in an *in vivo* system using a BMP inhibitor to see if a similar up-regulation of CCL20 is observed. Since CCL20 is known to recruit Th17 cells and modulate their immune response

(Comerford, Nibbs et al. 2010), we could extend these *in vitro* observations into a murine model that lacks CCR6, the receptor that is specific for binding with CCL20, to

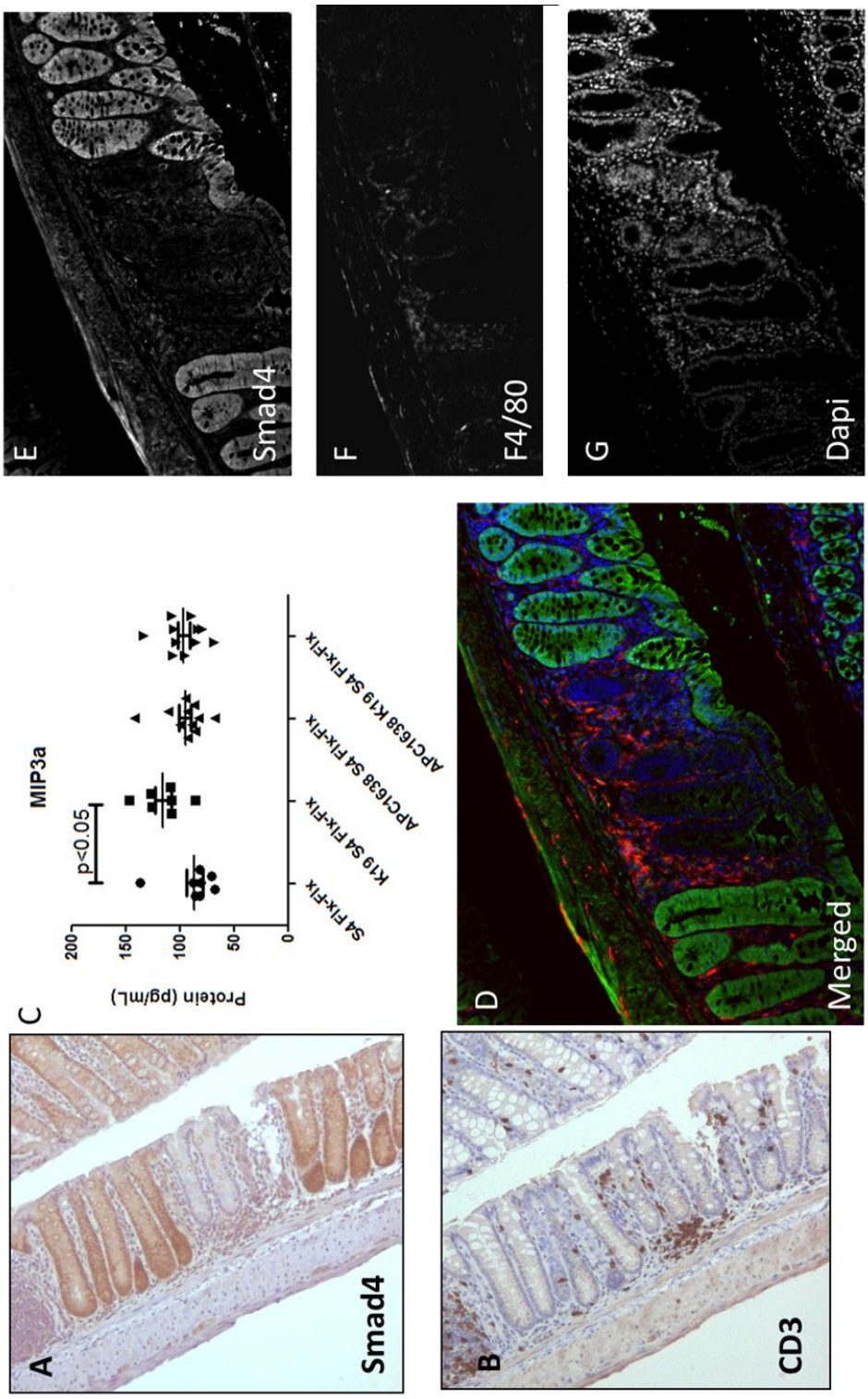


Figure 4.4. *Increased inflammatory Cells are Associated with Smad4 Loss.* (A) Smad4 stain (B) CD3+ stain in *matched sections* of K19Cre^{ERT2}Smad4$^{lox/lox}$ mouse colon which intestinal Smad4 loss has been induced by tamoxifen treatment. (C) Quantification of MIP3a results from Luminex multiplex assay. One-way Anova with Bon-ferroni posttest was performed to determine significance (n= 8 for Smad4$^{lox/lox}$; 7 for K19-Cre^{ERT2} Smad4$^{lox/lox}$; 11 for APC$^{1638/+}$Smad4$^{lox/lox}$; 11 for APC$^{1638/+}$K19Cre^{ERT2}Smad4$^{lox/lox}$) (D) Merged image of (E) Smad4 (Green) (F) F4/80 (Red) and (G) DAPI (Blue) of K19-Cre^{ERT2} Smad4$^{lox/lox}$ mouse one month post-tamoxifen treatment.

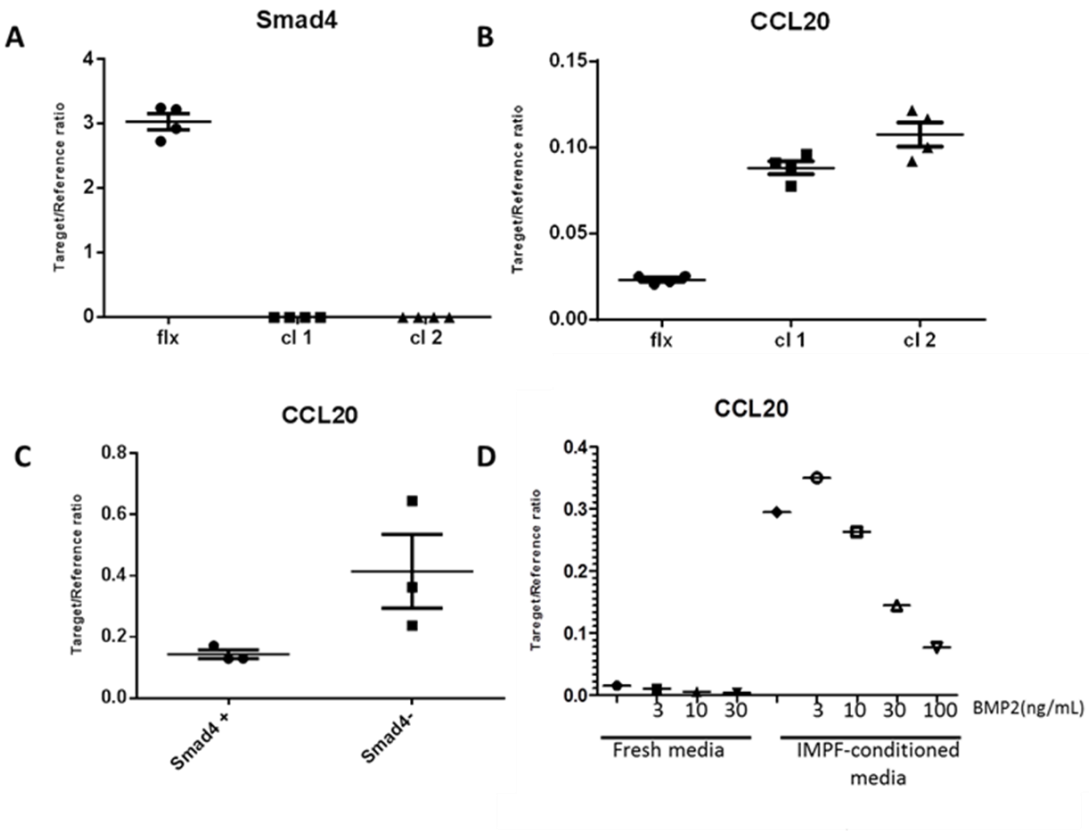


Figure 4.5. *Loss of Epithelial Smad4 increases expression of CCL20.* qPCR analysis of (A) Smad4 and (B) CCL20 from immortalized colonocytes deirived from CyclinD1-Smad4$^{flx/flx}$ mice. Colonocytes were either treated with Adeno-GFP (flx) or Adeno-Cre (cl1, cl2) before clonal selection for GFP expression or loss of Smad4 expression. (C) qPCR analysis of CCL20 expression from Lrig1Cre^{ERT} mice from either vehicle treated (Smad4+) or tamoxifen treated (Smad4-) specimens. (D) qPCR analysis (logarithmic scale) of Smad4 null colonocytes and Smad4 expressing colonocytes (flx) treated with BMP /IMPF conditioned media as indicated. Specimens were generated by Anna Means, Ph.D. while analysis was performed by Connie Weaver.

determine how loss of Smad4 influences immune response with and without the CCL20/CCR6 axis intact. We predict that without CCR6 that a Th17 response would be dampened. It would be interesting to extend these studies to our tumor models and observe how the CCL20/CCR6 axis affects tumor incidence and burden. We would predict that a dampened inflammatory response that there would be fewer tumors. However, Th17 cells have been shown to be both pro-inflammatory and immunosuppressive, so it would be interesting to observe what Th17 population is present in the context of loss of epithelial Smad4 expression.

Summary

Within the intestinal tract, a delicate balance between proliferation and differentiation must be maintained to ensure proper tissue function. Dysregulation of many pathways have been implicated in the pathogenesis of colorectal carcinoma; however, a large portion of CRC cases have mutation in the Wnt pathway (>80%) and a majority has a mutation within the BMP/TGF-β signaling pathway. Herein, we have generated a mouse model that recapitulates pathogenesis of colorectal cancer to gain insight into the role of a key transcription factor Smad4, the central mediator of TGF-β signaling pathway, plays in intestinal homeostasis. In the context of non-transformed intestinal epithelium, we observed an increase in proliferation, a decrease in mature colonocytes, and increased inflammation when Smad4 is lost. These findings are associated with an increase in Erk pathway activation and increased β-catenin mRNA but not increased Wnt pathway activation. In the context of transformed epithelium, we observed that Smad4 depletion leads to increased β-catenin mRNA and in the presence of APC mutation this resulted in an increase in canonical Wnt pathway activation and increased intestinal tumor burden. Overall, these results provide insights into the biology of intestinal homeostasis and into colorectal carcinoma progression.

APPENDIX

XIAP Monoubiquitylates Groucho/TLE to Promote Canonical Wnt Signaling

We collaborated with Allison Hanson and the Lee Laboratory in their study describing the role of XIAP, mammalian homologue of *Drosophila* Diap1, as a ubiquitin ligase for Groucho(Gro)/TLE (Hanson, Wallace et al. 2012). Hanson et al. identified Diap1 as an activator of Wingless signaling in *Drosophila* S2 cells and then went on to test the function of the mammalian XIAP in HEK293T and SW480 cell lines via siRNA knockdown. They found increased activity in TOP Flash, a reporter plasmid utilized to detect activation of canonical Wnt signaling when XIAP was reduced. For our part in this project, we perform qPCR analysis on Wnt target gene Axin2 upon depletion of XIAP (Figure A.1A Panel ii and Figure A.1B Panel ii) in HEK293T cells and SW480 cells. HEK293T cells were transfected with scrambled siRNA (Con) or siRNA targeting XIAP (as indicated in Figure A.1A Panel i) then cells were treated with exogenous Wnt3a for 24 hours in order to induce Wnt pathway activation. In cells that were treated with exogenous Wnt3a and control siRNA, we observed an increase in mRNA expression by qPCR (Lane 2, Figure A.1A Panel i) ; however, HEK293T cells that had XIAP depleted via siRNA transfection (Lanes 3-4, Figure A.1A Panel i) had Axin2 levels that were significantly reduced when compared to the Wnt3a stimulated cells treated with exogenous Wnt3a. XIAP depletion in SW480 cells showed a similar reduction in Axin2 expression (Figure A.1B Panel i) without need of exogenous Wnt treatment since SW480 cells have constitutively high levels of Wnt pathway activation. Hanson et al. went on to elegantly show that XIAP ubiquitilates Gro/TLE and this in turns disrupts Gro/TLE interaction with TCF. β-catenin then can bind with TCF and drive transcriptional program associated with activated canonical Wnt signaling.

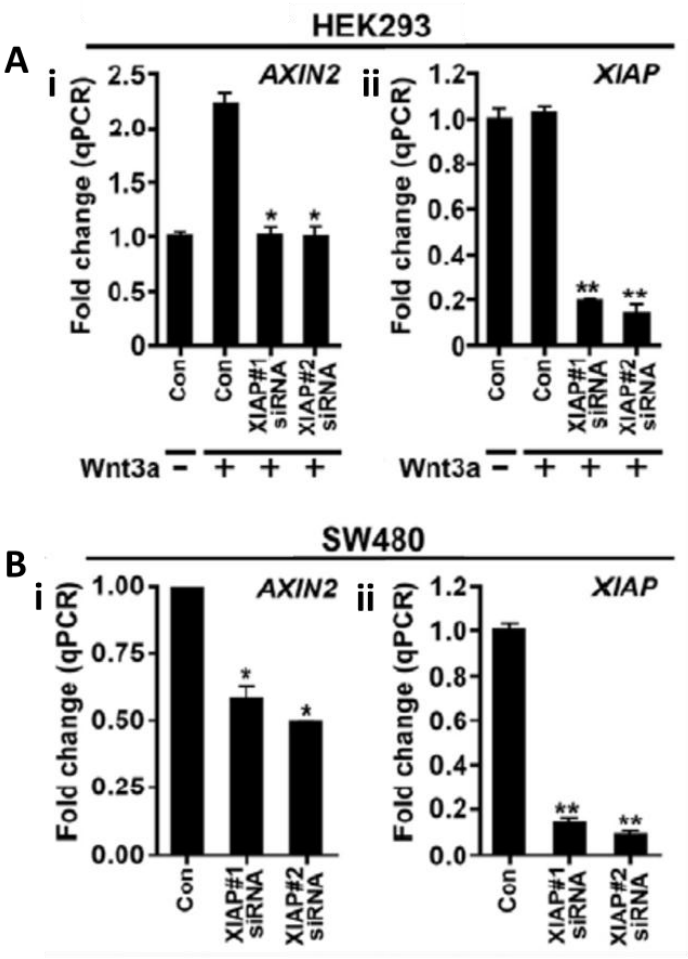


Figure A.1. *Knockdown of XIAP inhibits Wnt target gene expression*. Adapted from Hanson et al, 2012. Quantitative real-time PCR (qRT-PCR) analysis of Wnt target gene Axin2 expression (Panel i) in HEK293T (A) and SW480 (B) cells transfected with scrambled control (Con) siRNA or XIAP-targeting siRNA. Panel ii shows qRT-PCR analysis of XIAP expression in HEK293T (A) and SW480 (B) cells transfected with scrambled control (Con) siRNA or XIAP-targeting siRNA. Results (mean+/-SD) of four independent qRT-PCR reactions are displayed. *p-value < 0.001, **p-value <0.0001 compared to Wnt3a CM-treated non-targeting Con (A) or non-targeting Con (B).

Elevated ALCAM Shedding in Colorectal Cancer Correlates with Poor Patient Outcome

We collaborated with Amanda Hansen and the Zijlstra Laboratory in their work describing cleaved ALCAM correlating with poor colorectal cancer prognosis (Hansen, Freeman et al. 2013). Previous reports were contradictory as to whether overexpression of ALCAM or loss of ALCAM expression could function as a prognostic marker in colorectal cancer (Weichert, Knosel et al. 2004, Levin, Powell et al. 2010, Lugli, Iezzi et al. 2010, Tachezy, Zander et al. 2012). For our contribution to this work we provided microarray data from our 250 colorectal cancer patient dataset as well as the survival outcome associated with this patient dataset. We observed higher expression of ALCAM in colorectal cancer cases compared to normal tissue (Figure A.2A) and that higher than median expression of ALCAM associated with poor patient outcome (Figure A.2B). We also observed increased expression of ADAM17, the metalloprotease which cleaves ALCAM, in cancer cases (Figure A.2C); however, higher than median expression of ADAM17 did not significantly associate with poor patient prognosis (Figure A.2D). We also provided serum from colorectal patients for this study; however, levels of ALCAM within the serum did not significantly change in cancer patients when compared to normal controls (Figure A.2E) nor did increased expression correlate with stage of disease (Figure A.2F). Hansen et al. went on to develop an antibody to detect the intercellular domain of ALCAM, and when used with an antibody that detects the extracellular domain of ALCAM, it allowed Hansen et al. to define tissues samples which showed evidence of shed ALCAM. Upon examining tissue samples from Stage 2 colorectal cancer cases, they found that ALCAM shedding corresponded with poor patient outcome. This suggests that examining ALCAM expression alone may not be sufficient to predict patient outcome.

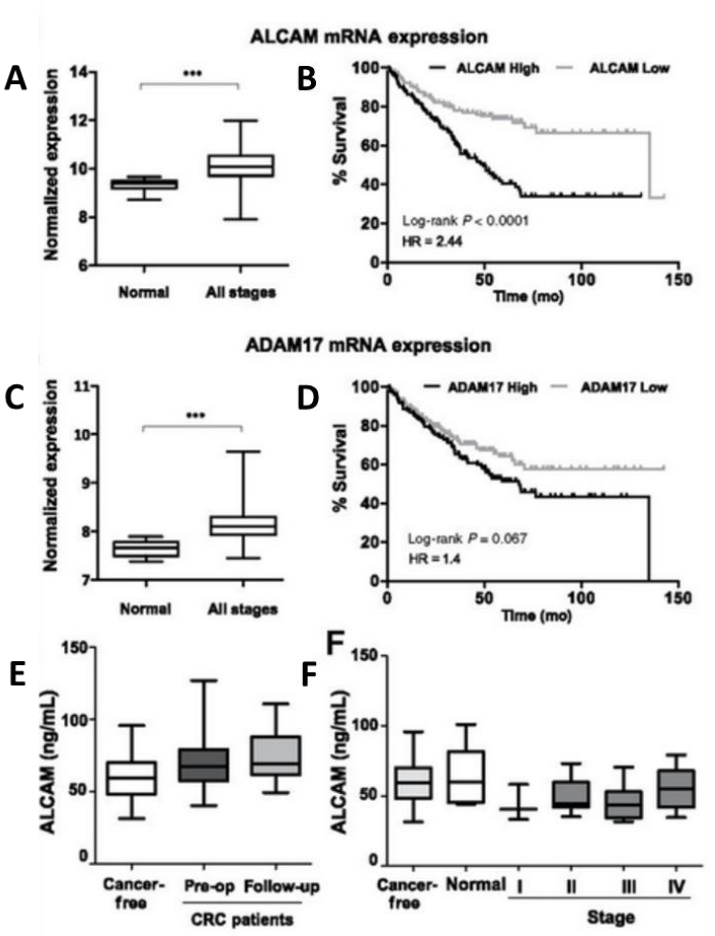


Figure A.2. *ALCAM expression in colorectal carcinoma and its correlation to patient outcome*. Adapted from Hansen et al, 2013. Expression (mRNA) of ALCAM (A) and ADAM17 (C) in a cohort of 250 patients with CRC was correlated to patient survival after dichotomizing the population across its mean expression into patients with "High ALCAM" and "Low ALCAM" (B) or "High ADAM17" and "Low ADAM17" (D). (E) Using an ALCAM ELISA, circulating levels of shed ALCAM were evaluated in serum from cancer-free patients (n = 48) and compared with serum from patients with CRC at time of diagnosis (pre-op; n = 42) and serum from patients at time of follow-up after treatment (post-op;n = 19). (F) Patients with CRC were stratified according to stage (I–IV) and compared with cancer-free patients (n = 48) and healthy adults (n = 6). Box plots show the mean, SD, and full range of the expression data. Survival is presented with Kaplan–Meier plots and log-rank test was used to evaluate significance. ***, $p < 0.001$.

Understanding the Role of NFAT in Colorectal Cancer

During my lab tenure, we developed an integrative and comparative computational approach to reveal transcriptional regulatory mechanisms underlying colon cancer progression in order to understand the mechanism/signaling pathways responsible for invasion, migration and metastasis in colorectal cancer. Our lab successfully modeled human cancer invasion/metastasis using mouse colon cancer cells (MC-38). We have applied this approach to fourteen human colorectal cancer (CRC) microarray data sets and to one microarray dataset from an immunocompetent mouse model of metastasis, and we have identified known and novel transcriptional regulators in CRC. Among these transcriptional regulators, the Nuclear Factor of Activated T cells (NFAT) family of transcription factors play a central role in inducible gene transcription in various signaling pathways including regulation of cell differentiation, development, adaption, immune system response, inflammation, adipocyte metabolism, and lipolysis, and carcinogenesis. We then turned to cell culture lines to study how the expression of specific NFAT family members alters certain biological aspects.

My focus for this project was the manipulation of NFATc2 in SW480 human CRC cell line. Upon depletion of NFATc2 (Figure A.3 D), we note that increased number of cells migrated through transwells compared to SW480 that were mock transfected or transfected with Scrambled non-targeting siRNA (Figure A.3A,B). We performed parallel proliferation studies to show that increased number of cells that were observed on the transwell was not due to an increase in proliferation when NFATc2 was knocked down (Figure A.3C). Later, in the project our focused shifted away from NFATc2 to NFATc1 as NFATc1 was the NFAT family member for which expression changed when MC-38^{parental}

cell line is compared MC-38^{met} cell line (a more highly metastatic derivative cell line) (Figure A.4). In data not included in this document, the tumor-associated NFATc1 co-regulated gene signature was associated with worse clinical survival outcomes in stage II colorectal cancer patients. RNAi-based inhibition of NFATc1 expression in the MC38^{met} cells resulted in decreased invasiveness in a transendothelial invasion model.

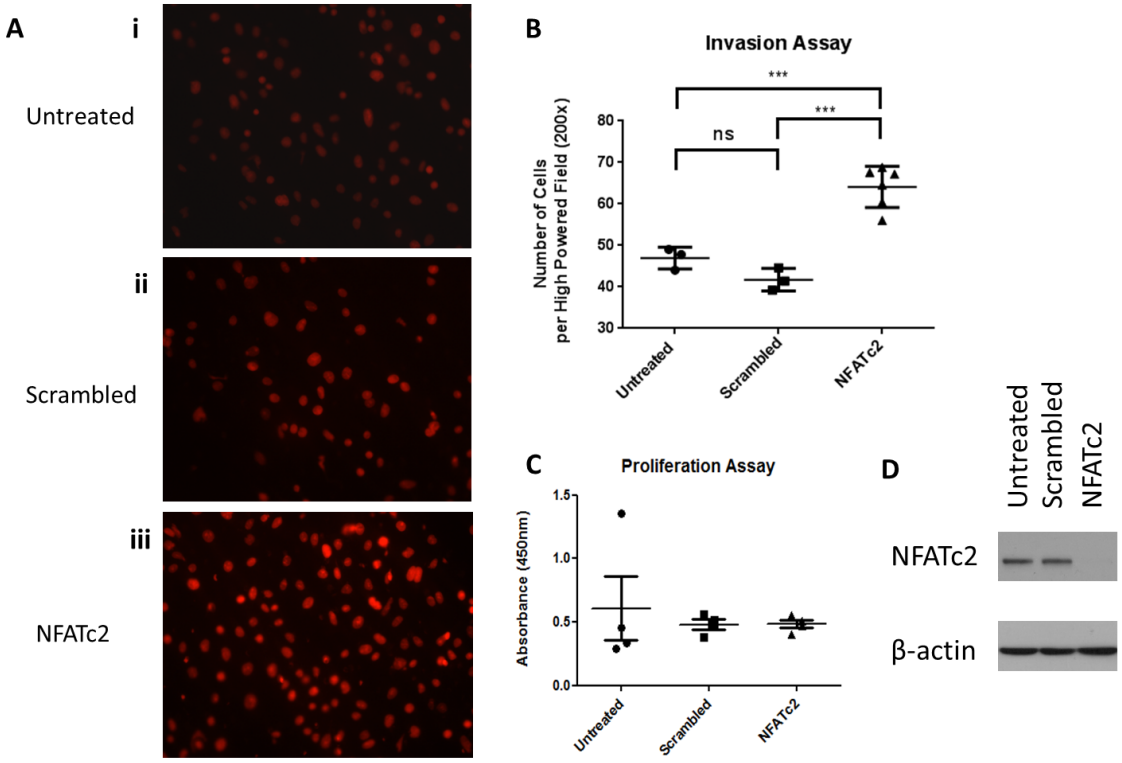


Figure A.3. *Knockdown of NFATc2 in SW480 cells is associated with Increased Invasion but not Increased proliferation.* (A) Representative high powered field (200x) of SW480 cells 24 hours after plating in matrigel on transwells. SW480 cells were either mock transfected (i) or with Scrambled siRNA (ii) or NFATc2-targeting siRNA 48 hours prior to plating. Transwells were fixed in 70% ethanol prior to being stained using Propidium Iodide. (B) Quantification of high power fields (hpfs) each dot represents an average of 5 hpfs on a separate transwell. Statistical significance as determined by ANOVA analysis with multiple comparisons post-test, *** = p<0.001, ns = not significant (C) Parallel WST-1 assay showing no significant difference in proliferation between mock, scrambled or NFATc2-targeting siRNA transfection. (D) Western blot analysis displaying effcient knockdown of NFATc2 48 hours post-transfection with β-actin serving as loading control.

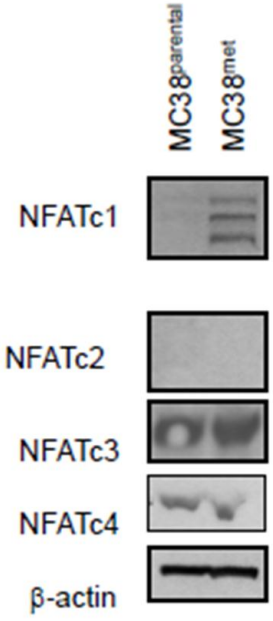


Figure A.4. *NFATc1 not NFATc2 is differentially expressed in cell line MC-38^{parental} compared to MC-38^{met}* . Western blot analysis displaying protein expression for NFATc1, NFATc2, NFATc3, and NFATc4 with β-actin serving as loading control. Western blot performed by Manish Tripathi, Ph.D.

Cathepsin B as a Driving Force of Esophageal Cell Invasion

In 70% of esophageal carcinoma cases, expression of TGFβ Receptor and E-cadherin is lost (Andl, Fargnoli et al. 2006). We collaborated with the Andl lab in their publication in which they found that invasion of esophageal carcinoma was dependent upon expression of Cathepsin B (Andl, McCowan et al. 2010). Using an organotypic culture model, they utilized TGFβ Receptor and E-cadherin dominant negative keratinocytes (EcdnT) and found that EcdnT invaded more so than their E-cadherin and TGFβ Receptor expressing counterparts. Examining the differential expression between the cell lines, they found that Cathepsin B was up-regulated in the EcdnT cell line. They went on to utilize short hairpin RNA (shRNA) targeting Cathepsin B to knockdown expression in the EcdnT cells. Upon depletion of Cathepsin B expression, EcdnT cells were less invasive.

For our contribution to the study, we aided in the data analysis of 80 tissue core samples taken from 40 esophageal carcinoma patients stained for expression of Cathepsin B, TGFβ Receptor and E-cadherin (Figure A.5A-D). Specifically, taking the data scored by another researcher, we performed the linear regression and showed an inverse association in patient samples (Figure A.5E). This finding validated the inverse relationship they observed in the EcdnT organotypic model recapitulates an aspect of the human esophageal carcinoma.

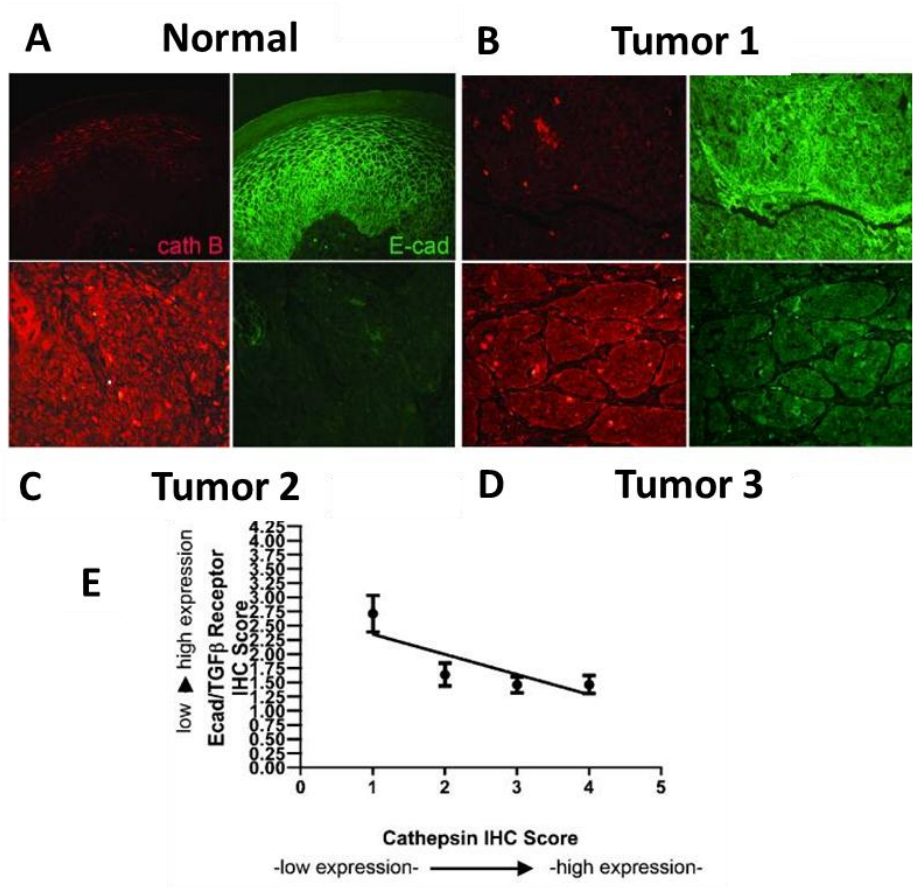


Figure A.5. *Cathepsin B protein expression is inversely associated with Ecad/TGFβ Receptor protein expression in human esophageal cancer patients.* Adapted from Andl et al 2010, Neoplasia. (A-D) Representative pictures of immunofluorescence staining for Cathepsin B and Ecad (A) Normal epithelium shows no signal for Cathepsin B (red, score 1) and a strong signal for Ecad (green, score 4) localizing to the membrane in normal esophageal epithelium. (B) Cathepsin B-negative tumor (red, score 1) has a strong signal for Ecad green, score 4, Tumor 1) (C) Cathepsin B-positive tumor is Ecad-negative (Tumor 2). (D) Intermediate expression levels that correspond to a score of 2 are shown for both molecules (Tumor 3) (E) Analysis of a tissue microarray with 80 esophageal tumor samples from 40 patients and 4 normal tissues. Tissues were stained and scored for Ecad, TβRII, and Cathepsin B expression. Scores ranged from 1 (absent) to 4 (highest expression). The graphic representation of the scores demonstrates the linear regression between Ecad/TβRII and Cathepsin B expression. Analysis using Spearman correlation shows a significant negative correlation (P = .0013, ρ = -0.34). Error bars, SEM.

Examining Wnt Signaling in Non-alcoholic Steatohepatitis

Non-alcoholic Steatohepatitis (NASH) affects some 2-5% of Americans and is associated with obesity and diabetes. Previously, research groups have associated Wnt signaling with being protective in this disease state as well as other liver ischemia models (Lehwald, Tao et al. 2011, Yang, Lee et al. 2014). Charles Flynn, Ph.D. noted a specific phospholipid pattern that occurred in his murine NASH model that was associated with activated Wnt signaling. To ascertain if there was a mouse model which showed localization of activation of Wnt signaling within the liver, he inquired if the Axin2 reporter mouse with β-galactosidase knocked in to the Axin2 locus would be a suitable model system. We collaborated with Dr. Flynn to generate preliminary data investigating if the Axin2 mouse would be a suitable model system. To this end, we performed whole mount staining on liver isolated from this mouse model and found that β-galactosidase activity was detected surrounding the central veins of the hepatic lobule (Figure A.6A,B). The phospholipid pattern Dr. Flynn noted in his NASH model had a similar pattern to β-galactosidase staining in the Axin2 mouse suggesting that the Axin2 knock-in mouse would be suitable to perform his studies.

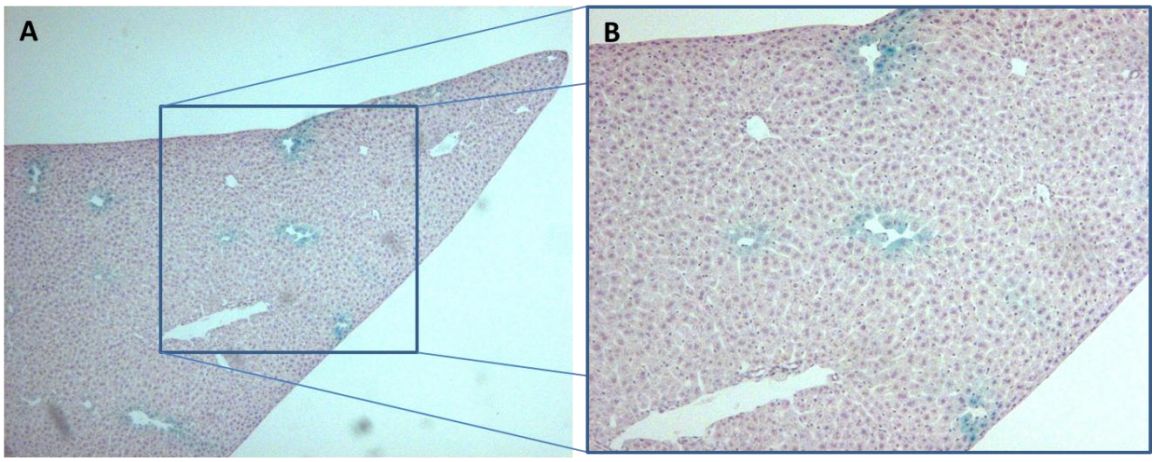


Figure A.6. *Axin-2 reporter mice show activation of Wnt signaling near the central vein of liver sections*. Photomicrographs of whole mount sections of Axin-2 β-galactosidase reporter mice after staining for β-galactosidase activity at (A) 50x and (B) 100x. This pattern suggests activation of canonical Wnt signaling can be detected within this model system.

REFERENCES

Alazzouzi, H., P. Alhopuro, R. Salovaara, H. Sammalkorpi, H. Jarvinen, J. P. Mecklin, A. Hemminki, S. Schwartz, Jr., L. A. Aaltonen and D. Arango (2005). "SMAD4 as a prognostic marker in colorectal cancer." Clin Cancer Res **11**(7): 2606-2611.

Andl, C. D., B. B. Fargnoli, T. Okawa, M. Bowser, M. Takaoka, H. Nakagawa, A. Klein-Szanto, X. Hua, M. Herlyn and A. K. Rustgi (2006). "Coordinated functions of E-cadherin and transforming growth factor beta receptor II in vitro and in vivo." Cancer Res **66**(20): 9878-9885.

Andl, C. D., K. M. McCowan, G. L. Allison and A. K. Rustgi (2010). "Cathepsin B is the driving force of esophageal cell invasion in a fibroblast-dependent manner." Neoplasia **12**(6): 485-498.

Aoki, K., F. Kakizaki, H. Sakashita, T. Manabe, M. Aoki and M. M. Taketo (2011). "Suppression of colonic polyposis by homeoprotein CDX2 through its nontranscriptional function that stabilizes p27Kip1." Cancer Res **71**(2): 593-602.

Arteaga, C. L., A. K. Tandon, D. D. Von Hoff and C. K. Osborne (1988). "Transforming growth factor beta: potential autocrine growth inhibitor of estrogen receptor-negative human breast cancer cells." Cancer Res **48**(14): 3898-3904.

Auclair, B. A., Y. D. Benoit, N. Rivard, Y. Mishina and N. Perreault (2007). "Bone morphogenetic protein signaling is essential for terminal differentiation of the intestinal secretory cell lineage." Gastroenterology **133**(3): 887-896.

Ayabe, T., D. P. Satchell, C. L. Wilson, W. C. Parks, M. E. Selsted and A. J. Ouellette (2000). "Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria." Nat Immunol **1**(2): 113-118.

Bakaris, S., A. Cetinkaya, F. Ezberci and H. Ekerbicer (2008). "Expression of homeodomain protein CDX2 in colorectal adenoma and adenocarcinoma." Histol Histopathol **23**(9): 1043-1047.

Bardeesy, N., K. H. Cheng, J. H. Berger, G. C. Chu, J. Pahler, P. Olson, A. F. Hezel, J. Horner, G. Y. Lauwers, D. Hanahan and R. A. DePinho (2006). "Smad4 is dispensable for normal pancreas development yet critical in progression and tumor biology of pancreas cancer." Genes Dev **20**(22): 3130-3146.

Barker, N., M. van de Wetering and H. Clevers (2008). "The intestinal stem cell." Genes Dev **22**(14): 1856-1864.

Barker, N., J. H. van Es, J. Kuipers, P. Kujala, M. van den Born, M. Cozijnsen, A. Haegebarth, J. Korving, H. Begthel, P. J. Peters and H. Clevers (2007). "Identification of stem cells in small intestine and colon by marker gene Lgr5." Nature **449**(7165): 1003-1007.

Barnard, J. A., R. D. Beauchamp, R. J. Coffey and H. L. Moses (1989). "Regulation of intestinal epithelial cell growth by transforming growth factor type beta." Proc Natl Acad Sci U S A **86**(5): 1578-1582.

Barnard, J. A., G. J. Warwick and L. I. Gold (1993). "Localization of transforming growth factor beta isoforms in the normal murine small intestine and colon." Gastroenterology **105**(1): 67-73.

Barry, D. P., M. Asim, B. P. Scull, M. B. Piazuelo, T. de Sablet, N. D. Lewis, L. A. Coburn, K. Singh, L. G. Ellies, A. P. Gobert, R. Chaturvedi and K. T. Wilson (2011). "Cationic amino acid transporter 2 enhances innate immunity during Helicobacter pylori infection." PLoS One **6**(12): e29046.

Bateman, N. W., D. Tan, R. G. Pestell, J. D. Black and A. R. Black (2004). "Intestinal tumor progression is associated with altered function of KLF5." J Biol Chem **279**(13): 12093-12101.

Beck, S. E., B. H. Jung, A. Fiorino, J. Gomez, E. Del Rosario, B. L. Cabrera, S. C. Huang, J. Y. C. Chow and J. M. Carethers (2006). "Bone morphogenetic protein signaling and growth suppression in colon cancer." American Journal of Physiology-Gastrointestinal and Liver Physiology **291**(1): G135-G145.

Beroud, C. and T. Soussi (1996). "APC gene: database of germline and somatic mutations in human tumors and cell lines." Nucleic Acids Res **24**(1): 121-124.
Biteau, B. and H. Jasper (2011). "EGF signaling regulates the proliferation of intestinal stem cells in Drosophila." Development **138**(6): 1045-1055.

Bos, J. L., E. R. Fearon, S. R. Hamilton, M. Verlaan-de Vries, J. H. van Boom, A. J. van der Eb and B. Vogelstein (1987). "Prevalence of ras gene mutations in human colorectal cancers." Nature **327**(6120): 293-297.

Brabletz, S., O. Schmalhofer and T. Brabletz (2009). "Gastrointestinal stem cells in development and cancer." J Pathol **217**(2): 307-317.

Brabletz, T., A. Jung, S. Reu, M. Porzner, F. Hlubek, L. A. Kunz-Schughart, R. Knuechel and T. Kirchner (2001). "Variable beta-catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment." Proc Natl Acad Sci U S A **98**(18): 10356-10361.

Buchon, N., N. A. Broderick, S. Chakrabarti and B. Lemaitre (2009). "Invasive and indigenous microbiota impact intestinal stem cell activity through multiple pathways in Drosophila." Genes Dev **23**(19): 2333-2344.

Buller, N. V., S. L. Rosekrans, J. Westerlund and G. R. van den Brink (2012). "Hedgehog signaling and maintenance of homeostasis in the intestinal epithelium." Physiology (Bethesda) **27**(3): 148-155.

Carmon, K. S., Q. Lin, X. Gong, A. Thomas and Q. Liu (2012). "LGR5 interacts and cointernalizes with Wnt receptors to modulate Wnt/beta-catenin signaling." Mol Cell Biol **32**(11): 2054-2064.

Cipriano, R., C. E. Kan, J. Graham, D. Danielpour, M. Stampfer and M. W. Jackson (2011). "TGF-beta signaling engages an ATM-CHK2-p53-independent RAS-induced senescence and prevents malignant transformation in human mammary epithelial cells." Proc Natl Acad Sci U S A **108**(21): 8668-8673.

Clarke, L. L. (2009). "A guide to Ussing chamber studies of mouse intestine." Am J Physiol Gastrointest Liver Physiol **296**(6): G1151-1166.

Clevers, H. (2006). "Wnt/beta-catenin signaling in development and disease." Cell **127**(3): 469-480.

Comerford, I., R. J. Nibbs, W. Litchfield, M. Bunting, Y. Harata-Lee, S. Haylock-Jacobs, S. Forrow, H. Korner and S. R. McColl (2010). "The atypical chemokine receptor CCX-CKR scavenges homeostatic chemokines in circulation and tissues and suppresses Th17 responses." Blood **116**(20): 4130-4140.

Costantini, F., E. H. Jho, T. Zhang, C. Domon, C. K. Joo and J. N. Freund (2002). "Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway." Molecular and Cellular Biology **22**(4): 1172-1183.

Creamer, B., R. G. Shorter and J. Bamforth (1961). "The turnover and shedding of epithelial cells. I. The turnover in the gastro-intestinal tract." Gut **2**: 110-118.

Cress, R. D., C. Morris, G. L. Ellison and M. T. Goodman (2006). "Secular changes in colorectal cancer incidence by subsite, stage at diagnosis, and race/ethnicity, 1992-2001." Cancer **107**(5 Suppl): 1142-1152.

Cross, D. A., D. R. Alessi, P. Cohen, M. Andjelkovich and B. A. Hemmings (1995). "Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B." Nature **378**(6559): 785-789.

Davies, H., G. R. Bignell, C. Cox, P. Stephens, S. Edkins, S. Clegg, J. Teague, H. Woffendin, M. J. Garnett, W. Bottomley, N. Davis, E. Dicks, R. Ewing, Y. Floyd, K. Gray, S. Hall, R. Hawes, J. Hughes, V. Kosmidou, A. Menzies, C. Mould, A. Parker, C. Stevens, S. Watt, S. Hooper, R. Wilson, H. Jayatilake, B. A. Gusterson, C. Cooper, J. Shipley, D. Hargrave, K. Pritchard-Jones, N. Maitland, G. Chenevix-Trench, G. J. Riggins, D. D. Bigner, G. Palmieri, A. Cossu, A. Flanagan, A. Nicholson, J. W. Ho, S. Y. Leung, S. T. Yuen, B. L. Weber, H. F. Seigler, T. L. Darrow, H. Paterson, R. Marais, C. J. Marshall, R. Wooster, M. R. Stratton and P. A. Futreal (2002). "Mutations of the BRAF gene in human cancer." Nature **417**(6892): 949-954.

de Lau, W., N. Barker, T. Y. Low, B. K. Koo, V. S. Li, H. Teunissen, P. Kujala, A. Haegebarth, P. J. Peters, M. van de Wetering, D. E. Stange, J. E. van Es, D. Guardavaccaro, R. B. Schasfoort, Y. Mohri, K. Nishimori, S. Mohammed, A. J. Heck and H. Clevers (2011). "Lgr5 homologues associate with Wnt receptors and mediate R-spondin signalling." Nature **476**(7360): 293-297.

de Lau, W., P. Kujala, K. Schneeberger, S. Middendorp, V. S. Li, N. Barker, A. Martens, F. Hofhuis, R. P. DeKoter, P. J. Peters, E. Nieuwenhuis and H. Clevers (2012). "Peyer's patch M cells derived from Lgr5(+) stem cells require SpiB and are induced by RankL in cultured "miniguts"." Mol Cell Biol **32**(18): 3639-3647.

de Lau, W. B., B. Snel and H. C. Clevers (2012). "The R-spondin protein family." Genome Biol **13**(3): 242.

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

Dhawan, P., A. B. Singh, N. G. Deane, Y. No, S. R. Shiou, C. Schmidt, J. Neff, M. K. Washington and R. D. Beauchamp (2005). "Claudin-1 regulates cellular transformation and metastatic behavior in colon cancer." J Clin Invest **115**(7): 1765-1776.

Edge, S. B. and C. C. Compton (2010). "The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM." Ann Surg Oncol **17**(6): 1471-1474.

Elliott, R. L. and G. C. Blobe (2005). "Role of transforming growth factor Beta in human cancer." J Clin Oncol **23**(9): 2078-2093.

Engel, M. E., M. A. McDonnell, B. K. Law and H. L. Moses (1999). "Interdependent SMAD and JNK signaling in transforming growth factor-beta-mediated transcription." J Biol Chem **274**(52): 37413-37420.

Eppert, K., S. W. Scherer, H. Ozcelik, R. Pirone, P. Hoodless, H. Kim, L. C. Tsui, B. Bapat, S. Gallinger, I. L. Andrulis, G. H. Thomsen, J. L. Wrana and L. Attisano (1996). "MADR2 maps to 18q21 and encodes a TGFbeta-regulated MAD-related protein that is functionally mutated in colorectal carcinoma." Cell **86**(4): 543-552.

Fearon, E. R. (2011). "Molecular genetics of colorectal cancer." Annu Rev Pathol **6**: 479-507.

Fearon, E. R. and B. Vogelstein (1990). "A genetic model for colorectal tumorigenesis." Cell **61**(5): 759-767.

Fevr, T., S. Robine, D. Louvard and J. Huelsken (2007). "Wnt/beta-catenin is essential for intestinal homeostasis and maintenance of intestinal stem cells." Mol Cell Biol **27**(21): 7551-7559.

Fodde, R. and T. Brabletz (2007). "Wnt/beta-catenin signaling in cancer stemness and malignant behavior." Curr Opin Cell Biol **19**(2): 150-158.

Fodde, R., W. Edelmann, K. Yang, C. van Leeuwen, C. Carlson, B. Renault, C. Breukel, E. Alt, M. Lipkin, P. M. Khan and et al. (1994). "A targeted chain-termination mutation in the mouse Apc gene results in multiple intestinal tumors." Proc Natl Acad Sci U S A **91**(19): 8969-8973.

Fre, S., M. Huyghe, P. Mourikis, S. Robine, D. Louvard and S. Artavanis-Tsakonas (2005). "Notch signals control the fate of immature progenitor cells in the intestine." Nature **435**(7044): 964-968.

Freeman, T. J., J. J. Smith, X. Chen, M. K. Washington, J. T. Roland, A. L. Means, S. A. Eschrich, T. J. Yeatman, N. G. Deane and R. D. Beauchamp (2012). "Smad4-mediated signaling inhibits intestinal neoplasia by inhibiting expression of beta-catenin." Gastroenterology **142**(3): 562-571 e562.

Fritzmann, J., M. Morkel, D. Besser, J. Budczies, F. Kosel, F. H. Brembeck, U. Stein, I. Fichtner, P. M. Schlag and W. Birchmeier (2009). "A colorectal cancer expression profile that includes transforming growth factor beta inhibitor BAMBI predicts metastatic potential." Gastroenterology **137**(1): 165-175.

Fu, J., B. Wei, T. Wen, M. E. Johansson, X. Liu, E. Bradford, K. A. Thomsson, S. McGee, L. Mansour, M. Tong, J. M. McDaniel, T. J. Sferra, J. R. Turner, H. Chen, G. C. Hansson, J. Braun and L. Xia (2011). "Loss of intestinal core 1-derived O-glycans causes spontaneous colitis in mice." J Clin Invest **121**(4): 1657-1666.

Fujimura, Y. and M. Iida (2001). "A new marker for cup cells in the rabbit small intestine: expression of vimentin intermediate filament protein." Med Electron Microsc **34**(4): 223-229.

Fuller, M. K., D. M. Faulk, N. Sundaram, M. M. Mahe, K. M. Stout, R. J. von Furstenberg, B. J. Smith, K. K. McNaughton, N. F. Shroyer, M. A. Helmrath and S. J. Henning (2013). "Intestinal stem cells remain viable after prolonged tissue storage." Cell Tissue Res **354**(2): 441-450.

Galgano, M. T., G. M. Hampton and H. F. Frierson, Jr. (2006). "Comprehensive analysis of HE4 expression in normal and malignant human tissues." Mod Pathol **19**(6): 847-853.
Gao, N., P. White and K. H. Kaestner (2009). "Establishment of intestinal identity and epithelial-mesenchymal signaling by Cdx2." Dev Cell **16**(4): 588-599.

Garabedian, E. M., L. J. Roberts, M. S. McNevin and J. I. Gordon (1997). "Examining the role of Paneth cells in the small intestine by lineage ablation in transgenic mice." J Biol Chem **272**(38): 23729-23740.

Garrett, T. P., N. M. McKern, M. Lou, T. C. Elleman, T. E. Adams, G. O. Lovrecz, H. J. Zhu, F. Walker, M. J. Frenkel, P. A. Hoyne, R. N. Jorissen, E. C. Nice, A. W. Burgess and C. W. Ward (2002). "Crystal structure of a truncated epidermal growth factor receptor extracellular domain bound to transforming growth factor alpha." Cell **110**(6): 763-773.

Gerbe, F., C. Legraverend and P. Jay (2012). "The intestinal epithelium tuft cells: specification and function." Cell Mol Life Sci **69**(17): 2907-2917.

Gerbe, F., J. H. van Es, L. Makrini, B. Brulin, G. Mellitzer, S. Robine, B. Romagnolo, N. F. Shroyer, J. F. Bourgaux, C. Pignodel, H. Clevers and P. Jay (2011). "Distinct ATOH1 and Neurog3 requirements define tuft cells as a new secretory cell type in the intestinal epithelium." J Cell Biol **192**(5): 767-780.

Glinka, A., C. Dolde, N. Kirsch, Y. L. Huang, O. Kazanskaya, D. Ingelfinger, M. Boutros, C. M. Cruciat and C. Niehrs (2011). "LGR4 and LGR5 are R-spondin receptors mediating Wnt/beta-catenin and Wnt/PCP signalling." EMBO Rep **12**(10): 1055-1061.

Grady, W. M., L. L. Myeroff, S. E. Swinler, A. Rajput, S. Thiagalingam, J. D. Lutterbaugh, A. Neumann, M. G. Brattain, J. Chang, S. J. Kim, K. W. Kinzler, B. Vogelstein, J. K. Willson and S. Markowitz (1999). "Mutational inactivation of transforming growth factor beta receptor type II in microsatellite stable colon cancers." Cancer Res **59**(2): 320-324.

Grainger, S., J. G. Savory and D. Lohnes (2010). "Cdx2 regulates patterning of the intestinal epithelium." Dev Biol **339**(1): 155-165.
Gregorieff, A. and H. Clevers (2010). "In situ hybridization to identify gut stem cells." Curr Protoc Stem Cell Biol **Chapter 2**: Unit 2F 1.

Grossmann, J., K. Walther, M. Artinger, P. Rummele, M. Woenckhaus and J. Scholmerich (2002). "Induction of apoptosis before shedding of human intestinal epithelial cells." Am J Gastroenterol **97**(6): 1421-1428.

Gryfe, R., H. Kim, E. T. Hsieh, M. D. Aronson, E. J. Holowaty, S. B. Bull, M. Redston and S. Gallinger (2000). "Tumor microsatellite instability and clinical outcome in young patients with colorectal cancer." N Engl J Med **342**(2): 69-77.

Guillemot, F., A. Nagy, A. Auerbach, J. Rossant and A. L. Joyner (1994). "Essential role of Mash-2 in extraembryonic development." Nature **371**(6495): 333-336.
Gupta, G. P. and J. Massague (2006). "Cancer metastasis: building a framework." Cell **127**(4): 679-695.

Guruharsha, K. G., M. W. Kankel and S. Artavanis-Tsakonas (2012). "The Notch signalling system: recent insights into the complexity of a conserved pathway." Nat Rev Genet **13**(9): 654-666.

Han, S. U., H. T. Kim, D. H. Seong, Y. S. Kim, Y. S. Park, Y. J. Bang, H. K. Yang and S. J. Kim (2004). "Loss of the Smad3 expression increases susceptibility to tumorigenicity in human gastric cancer." Oncogene **23**(7): 1333-1341.

Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." Cell **100**(1): 57-70.
Hanahan, D. and R. A. Weinberg (2011). "Hallmarks of cancer: the next generation." Cell **144**(5): 646-674.

Hansen, A. G., T. J. Freeman, S. A. Arnold, A. Starchenko, C. R. Jones-Paris, M. A. Gilger, M. K. Washington, K. H. Fan, Y. Shyr, R. D. Beauchamp and A. Zijlstra (2013). "Elevated ALCAM shedding in colorectal cancer correlates with poor patient outcome." Cancer Res **73**(10): 2955-2964.

Hanson, A. J., H. A. Wallace, T. J. Freeman, R. D. Beauchamp, L. A. Lee and E. Lee (2012). "XIAP monoubiquitylates Groucho/TLE to promote canonical Wnt signaling." Mol Cell **45**(5): 619-628.

Haramis, A. P., H. Begthel, M. van den Born, J. van Es, S. Jonkheer, G. J. Offerhaus and H. Clevers (2004). "De novo crypt formation and juvenile polyposis on BMP inhibition in mouse intestine." Science **303**(5664): 1684-1686.

Hardwick, J. C., G. R. Van Den Brink, S. A. Bleuming, I. Ballester, J. M. Van Den Brande, J. J. Keller, G. J. Offerhaus, S. J. Van Deventer and M. P. Peppelenbosch (2004). "Bone morphogenetic protein 2 is expressed by, and acts upon, mature epithelial cells in the colon." Gastroenterology **126**(1): 111-121.

Hardwick, J. C. H., G. R. Van den Brink, S. A. Bleuming, I. Ballester, J. M. H. Van den Brande, J. J. Keller, G. J. A. Offerhaus, S. J. H. Van Deventer and M. P. Peppelenbosch

(2004). "Bone morphogenetic protein 2 is expressed by, and acts upon, mature epithelial cells in the colon." Gastroenterology **126**(1): 111-121.

Hata, A., G. Lagna, J. Massague and A. Hemmati-Brivanlou (1998). "Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor." Genes Dev **12**(2): 186-197.

Hawk, E. T. and B. Levin (2005). "Colorectal cancer prevention." J Clin Oncol **23**(2): 378-391.

Hayashida, T., M. Decaestecker and H. W. Schnaper (2003). "Cross-talk between ERK MAP kinase and Smad signaling pathways enhances TGF-beta-dependent responses in human mesangial cells." FASEB J **17**(11): 1576-1578.

He, T. C., A. B. Sparks, C. Rago, H. Hermeking, L. Zawel, L. T. da Costa, P. J. Morin, B. Vogelstein and K. W. Kinzler (1998). "Identification of c-MYC as a target of the APC pathway." Science **281**(5382): 1509-1512.

He, X. C., J. Zhang, W. G. Tong, O. Tawfik, J. Ross, D. H. Scoville, Q. Tian, X. Zeng, X. He, L. M. Wiedemann, Y. Mishina and L. Li (2004). "BMP signaling inhibits intestinal stem cell self-renewal through suppression of Wnt-beta-catenin signaling." Nat Genet **36**(10): 1117-1121.

Hinoi, T., M. Loda and E. R. Fearon (2003). "Silencing of CDX2 expression in colon cancer via a dominant repression pathway." J Biol Chem **278**(45): 44608-44616.

Hirsinger, E., D. Duprez, C. Jouve, P. Malapert, J. Cooke and O. Pourquie (1997). "Noggin acts downstream of Wnt and Sonic Hedgehog to antagonize BMP4 in avian somite patterning." Development **124**(22): 4605-4614.

Hofer, D. and D. Drenckhahn (1996). "Cytoskeletal markers allowing discrimination between brush cells and other epithelial cells of the gut including enteroendocrine cells." Histochem Cell Biol **105**(5): 405-412.

Hoffmann, F. M. and S. K. Lim (2006). "Smad4 cooperates with lymphoid enhancer-binding factor 1/T cell-specific factor to increase c-myc expression in the absence of TGF-beta signaling." Proceedings of the National Academy of Sciences of the United States of America **103**(49): 18580-18585.

Hoosein, N. M., M. K. McKnight, A. E. Levine, K. M. Mulder, K. E. Childress, D. E. Brattain and M. G. Brattain (1989). "Differential sensitivity of subclasses of human colon carcinoma cell lines to the growth inhibitory effects of transforming growth factor-beta 1." Exp Cell Res **181**(2): 442-453.

Hough, C., M. Radu and J. J. Dore (2012). "Tgf-beta induced Erk phosphorylation of smad linker region regulates smad signaling." PLoS One **7**(8): e42513.

Howe, J. R., S. Roth, J. C. Ringold, R. W. Summers, H. J. Jarvinen, P. Sistonen, I. P. Tomlinson, R. S. Houlston, S. Bevan, F. A. Mitros, E. M. Stone and L. A. Aaltonen (1998). "Mutations in the SMAD4/DPC4 gene in juvenile polyposis." Science **280**(5366): 1086-1088.

Howe, J. R., M. G. Sayed, A. F. Ahmed, J. Ringold, J. Larsen-Haidle, A. Merg, F. A. Mitros, C. A. Vaccaro, G. M. Petersen, F. M. Giardiello, S. T. Tinley, L. A. Aaltonen and H. T. Lynch (2004). "The prevalence of MADH4 and BMPR1A mutations in juvenile polyposis and absence of BMPR2, BMPR1B, and ACVR1 mutations." J Med Genet **41**(7): 484-491.

Isaksson-Mettavainio, M., R. Palmqvist, J. Forssell, R. Stenling and A. Oberg (2006). "SMAD4/DPC4 expression and prognosis in human colorectal cancer." Anticancer Res **26**(1B): 507-510.

Jho, E. H., T. Zhang, C. Domon, C. K. Joo, J. N. Freund and F. Costantini (2002). "Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway." Mol Cell Biol **22**(4): 1172-1183.

Jubb, A. M., S. Chalasani, G. D. Frantz, R. Smits, H. I. Grabsch, V. Kavi, N. J. Maughan, K. J. Hillan, P. Quirke and H. Koeppen (2006). "Achaete-scute like 2 (ascl2) is a target of Wnt signalling and is upregulated in intestinal neoplasia." Oncogene **25**(24): 3445-3457.

Kim, B. G., C. Li, W. Qiao, M. Mamura, B. Kasprzak, M. Anver, L. Wolfraim, S. Hong, E. Mushinski, M. Potter, S. J. Kim, X. Y. Fu, C. Deng and J. J. Letterio (2006). "Smad4 signalling in T cells is required for suppression of gastrointestinal cancer." Nature **441**(7096): 1015-1019.

Kim, H. S. (2006). "Discriminative factor analysis of juvenile delinquency in South Korea." Taehan Kanho Hakhoe Chi **36**(8): 1315-1323.

Kim, K. K., Y. Wei, C. Szekeres, M. C. Kugler, P. J. Wolters, M. L. Hill, J. A. Frank, A. N. Brumwell, S. E. Wheeler, J. A. Kreidberg and H. A. Chapman (2009). "Epithelial cell alpha3beta1 integrin links beta-catenin and Smad signaling to promote myofibroblast formation and pulmonary fibrosis." J Clin Invest **119**(1): 213-224.

Kimchi, A., X. F. Wang, R. A. Weinberg, S. Cheifetz and J. Massague (1988). "Absence of TGF-beta receptors and growth inhibitory responses in retinoblastoma cells." Science **240**(4849): 196-199.

Kinzler, K. W., T. C. He, A. B. Sparks, C. Rago, H. Hermeking, L. Zawel, L. T. da Costa, P. J. Morin and B. Vogelstein (1998). "Identification of c-MYC as a target of the APC pathway." Science **281**(5382): 1509-1512.

Kinzler, K. W. and B. Vogelstein (1996). "Lessons from hereditary colorectal cancer." Cell **87**(2): 159-170.

Kleeff, J., H. Maruyama, T. Ishiwata, H. Sawhney, H. Friess, M. W. Buchler and M. Korc (1999). "Bone morphogenetic protein 2 exerts diverse effects on cell growth in vitro and is expressed in human pancreatic cancer in vivo." Gastroenterology **116**(5): 1202-1216.

Knudson, A. G. (1971). "Mutation and Cancer - Statistical Study of Retinoblastoma." Proceedings of the National Academy of Sciences of the United States of America **68**(4): 820-&.

Ko, T. C., H. M. Sheng, D. Reisman, E. A. Thompson and R. D. Beauchamp (1995). "Transforming growth factor-beta 1 inhibits cyclin D1 expression in intestinal epithelial cells." Oncogene **10**(1): 177-184.

Ko, T. C., W. Yu, T. Sakai, H. Sheng, J. Shao, R. D. Beauchamp and E. A. Thompson (1998). "TGF-beta1 effects on proliferation of rat intestinal epithelial cells are due to inhibition of cyclin D1 expression." Oncogene **16**(26): 3445-3454.

Kodach, L. L., S. A. Bleuming, A. R. Musler, M. P. Peppelenbosch, D. W. Hommes, G. R. van den Brink, C. J. van Noesel, G. J. Offerhaus and J. C. Hardwick (2008). "The bone morphogenetic protein pathway is active in human colon adenomas and inactivated in colorectal cancer." Cancer **112**(2): 300-306.

Kolterud, A., A. S. Grosse, W. J. Zacharias, K. D. Walton, K. E. Kretovich, B. B. Madison, M. Waghray, J. E. Ferris, C. Hu, J. L. Merchant, A. A. Dlugosz, A. H. Kottmann and D. L. Gumucio (2009). "Paracrine Hedgehog signaling in stomach and intestine: new roles for hedgehog in gastrointestinal patterning." Gastroenterology **137**(2): 618-628.

Kosinski, C., V. S. Li, A. S. Chan, J. Zhang, C. Ho, W. Y. Tsui, T. L. Chan, R. C. Mifflin, D. W. Powell, S. T. Yuen, S. Y. Leung and X. Chen (2007). "Gene expression patterns of human colon tops and basal crypts and BMP antagonists as intestinal stem cell niche factors." Proc Natl Acad Sci U S A **104**(39): 15418-15423.

Kosinski, C., D. E. Stange, C. Xu, A. S. Chan, C. Ho, S. T. Yuen, R. C. Mifflin, D. W. Powell, H. Clevers, S. Y. Leung and X. Chen (2010). "Indian hedgehog regulates intestinal stem cell fate through epithelial-mesenchymal interactions during development." Gastroenterology **139**(3): 893-903.

Krueger, F., Z. Madeja, M. Hemberger, M. McMahon, S. J. Cook and S. J. Gaunt (2009). "Down-regulation of Cdx2 in colorectal carcinoma cells by the Raf-MEK-ERK 1/2 pathway." Cell Signal **21**(12): 1846-1856.

Kuhn, R., F. Schwenk, M. Aguet and K. Rajewsky (1995). "Inducible gene targeting in mice." Science **269**(5229): 1427-1429.

Kurokowa, M., K. Lynch and D. K. Podolsky (1987). "Effects of growth factors on an intestinal epithelial cell line: transforming growth factor beta inhibits proliferation and stimulates differentiation." Biochem Biophys Res Commun **142**(3): 775-782.

Labbe, E., A. Letamendia and L. Attisano (2000). "Association of Smads with lymphoid enhancer binding factor 1/T cell-specific factor mediates cooperative signaling by the transforming growth factor-beta and wnt pathways." Proc Natl Acad Sci U S A **97**(15): 8358-8363.

Lassmann, S., R. Weis, F. Makowiec, J. Roth, M. Danciu, U. Hopt and M. Werner (2007). "Array CGH identifies distinct DNA copy number profiles of oncogenes and tumor suppressor genes in chromosomal- and microsatellite-unstable sporadic colorectal carcinomas." J Mol Med **85**(3): 293-304.

Laurent-Puig, P., C. Beroud and T. Soussi (1998). "APC gene: database of germline and somatic mutations in human tumors and cell lines." Nucleic Acids Res **26**(1): 269-270.

Laurent-Puig, P., A. Cayre, G. Manceau, E. Buc, J. B. Bachet, T. Lecomte, P. Rougier, A. Lievre, B. Landi, V. Boige, M. Ducreux, M. Ychou, F. Bibeau, O. Bouche, J. Reid, S. Stone and F. Penault-Llorca (2009). "Analysis of PTEN, BRAF, and EGFR status in determining benefit from cetuximab therapy in wild-type KRAS metastatic colon cancer." J Clin Oncol **27**(35): 5924-5930.

Lehwald, N., G. Z. Tao, K. Y. Jang, M. Sorkin, W. T. Knoefel and K. G. Sylvester (2011). "Wnt-beta-catenin signaling protects against hepatic ischemia and reperfusion injury in mice." Gastroenterology **141**(2): 707-718, 718 e701-705.

Lemieux, E., M. J. Boucher, S. Mongrain, F. Boudreau, C. Asselin and N. Rivard (2011). "Constitutive activation of the MEK/ERK pathway inhibits intestinal epithelial cell differentiation." Am J Physiol Gastrointest Liver Physiol **301**(4): G719-730.

Levin, T. G., A. E. Powell, P. S. Davies, A. D. Silk, A. D. Dismuke, E. C. Anderson, J. R. Swain and M. H. Wong (2010). "Characterization of the intestinal cancer stem cell marker CD166 in the human and mouse gastrointestinal tract." Gastroenterology **139**(6): 2072-2082 e2075.

Leystra, A. A., D. A. Deming, C. D. Zahm, M. Farhoud, T. J. Olson, J. N. Hadac, L. A. Nettekoven, D. M. Albrecht, L. Clipson, R. Sullivan, M. K. Washington, J. R. Torrealba, J. P. Weichert and R. B. Halberg (2012). "Mice expressing activated PI3K rapidly develop advanced colon cancer." Cancer Res **72**(12): 2931-2936.

Li, J., X. Huang, X. Xu, J. Mayo, P. Bringas, Jr., R. Jiang, S. Wang and Y. Chai (2011). "SMAD4-mediated WNT signaling controls the fate of cranial neural crest cells during tooth morphogenesis." Development.

Li, L. H., X. C. He, J. W. Zhang, W. G. Tong, O. Tawfik, J. Ross, D. H. Scoville, Q. Tian, X. Zeng, X. He, L. M. Wiedemann and Y. Mishina (2004). "BMP signaling inhibits intestinal stem cell self-renewal through suppression of Wnt-beta-catenin signaling." Nature Genetics **36**(10): 1117-1121.

Li, W., T. Zhu and K. L. Guan (2004). "Transformation potential of Ras isoforms correlates with activation of phosphatidylinositol 3-kinase but not ERK." J Biol Chem **279**(36): 37398-37406.

Linggi, B. and G. Carpenter (2006). "ErbB receptors: new insights on mechanisms and biology." Trends Cell Biol **16**(12): 649-656.

Liu, Q., Y. Zhang, H. Mao, W. Chen, N. Luo, Q. Zhou, W. Chen and X. Yu (2012). "A crosstalk between the Smad and JNK signaling in the TGF-beta-induced epithelial-mesenchymal transition in rat peritoneal mesothelial cells." PLoS One **7**(2): e32009.

Liu, X., R. Lu, S. Wu and J. Sun (2010). "Salmonella regulation of intestinal stem cells through the Wnt/beta-catenin pathway." FEBS Lett **584**(5): 911-916.

Logan, C. Y. and R. Nusse (2004). "The Wnt signaling pathway in development and disease." Annu Rev Cell Dev Biol **20**: 781-810.

Lugli, A., G. Iezzi, I. Hostettler, M. G. Muraro, V. Mele, L. Tornillo, V. Carafa, G. Spagnoli, L. Terracciano and I. Zlobec (2010). "Prognostic impact of the expression of putative cancer stem cell markers CD133, CD166, CD44s, EpCAM, and ALDH1 in colorectal cancer." Br J Cancer **103**(3): 382-390.

Madara, J. L. (1982). "Cup cells: structure and distribution of a unique class of epithelial cells in guinea pig, rabbit, and monkey small intestine." Gastroenterology **83**(5): 981-994.

Madara, J. L. (1989). "Loosening tight junctions. Lessons from the intestine." J Clin Invest **83**(4): 1089-1094.

Madara, J. L. and M. A. Marcial (1984). "Structural correlates of intestinal tight-junction permeability." Kroc Found Ser **17**: 77-100.

Manning, A. M., A. C. Williams, S. M. Game and C. Paraskeva (1991). "Differential sensitivity of human colonic adenoma and carcinoma cells to transforming growth factor beta (TGF-beta): conversion of an adenoma cell line to a tumorigenic phenotype is accompanied by a reduced response to the inhibitory effects of TGF-beta." Oncogene **6**(8): 1471-1476.

Marcelino, J., C. M. Sciortino, M. F. Romero, L. M. Ulatowski, R. T. Ballock, A. N. Economides, P. M. Eimon, R. M. Harland and M. L. Warman (2001). "Human disease-causing NOG missense mutations: effects on noggin secretion, dimer formation, and bone morphogenetic protein binding." Proc Natl Acad Sci U S A **98**(20): 11353-11358.

Markowitz, S., J. Wang, L. Myeroff, R. Parsons, L. Sun, J. Lutterbaugh, R. S. Fan, E. Zborowska, K. W. Kinzler, B. Vogelstein and et al. (1995). "Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability." Science **268**(5215): 1336-1338.

Massague, J. (2008). "TGFbeta in Cancer." Cell **134**(2): 215-230.

Massague, J., J. Seoane and D. Wotton (2005). "Smad transcription factors." Genes Dev **19**(23): 2783-2810.

Means, A. L., Y. Xu, A. Zhao, K. C. Ray and G. Gu (2008). "A CK19(CreERT) knockin mouse line allows for conditional DNA recombination in epithelial cells in multiple endodermal organs." Genesis **46**(6): 318-323.

Merlos-Suarez, A., F. M. Barriga, P. Jung, M. Iglesias, M. V. Cespedes, D. Rossell, M. Sevillano, X. Hernando-Momblona, V. da Silva-Diz, P. Munoz, H. Clevers, E. Sancho, R. Mangues and E. Batlle (2011). "The intestinal stem cell signature identifies colorectal cancer stem cells and predicts disease relapse." Cell Stem Cell **8**(5): 511-524.

Mesker, W. E., G. J. Liefers, J. M. Junggeburt, G. W. van Pelt, P. Alberici, P. J. Kuppen, N. F. Miranda, K. A. van Leeuwen, H. Morreau, K. Szuhai, R. A. Tollenaar and H. J. Tanke (2009). "Presence of a high amount of stroma and downregulation of SMAD4 predict for worse survival for stage I-II colon cancer patients." Cell Oncol **31**(3): 169-178.

Miyaki, M., T. Iijima, M. Konishi, K. Sakai, A. Ishii, M. Yasuno, T. Hishima, M. Koike, N. Shitara, T. Iwama, J. Utsunomiya, T. Kuroki and T. Mori (1999). "Higher frequency of Smad4 gene mutation in human colorectal cancer with distant metastasis." Oncogene **18**(20): 3098-3103.

Molenaar, M., M. vandeWetering, M. Oosterwegel, J. PetersonMaduro, S. Godsave, V. Korinek, J. Roose, O. Destree and H. Clevers (1996). "XTcf-3 transcription factor mediates beta-catenin-induced axis formation in Xenopus embryos." Cell **86**(3): 391-399.

Montgomery, R. K., D. L. Carlone, C. A. Richmond, L. Farilla, M. E. Kranendonk, D. E. Henderson, N. Y. Baffour-Awuah, D. M. Ambruzs, L. K. Fogli, S. Algra and D. T. Breault (2011). "Mouse telomerase reverse transcriptase (mTert) expression marks slowly cycling intestinal stem cells." Proc Natl Acad Sci U S A **108**(1): 179-184.

Moran, G. W., F. C. Leslie, S. E. Levison, J. Worthington and J. T. McLaughlin (2008). "Enteroendocrine cells: neglected players in gastrointestinal disorders?" Therap Adv Gastroenterol **1**(1): 51-60.

Morin, P. J., A. B. Sparks, V. Korinek, N. Barker, H. Clevers, B. Vogelstein and K. W. Kinzler (1997). "Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC." Science **275**(5307): 1787-1790.

Moser, A. R., H. C. Pitot and W. F. Dove (1990). "A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse." Science **247**(4940): 322-324.

Nakao, A., M. Afrakhte, A. Moren, T. Nakayama, J. L. Christian, R. Heuchel, S. Itoh, M. Kawabata, N. E. Heldin, C. H. Heldin and P. ten Dijke (1997). "Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling." Nature **389**(6651): 631-635.

Nam, K. T., H. J. Lee, J. J. Smith, L. A. Lapierre, V. P. Kamath, X. Chen, B. J. Aronow, T. J. Yeatman, S. G. Bhartur, B. C. Calhoun, B. Condie, N. R. Manley, R. D. Beauchamp, R. J. Coffey and J. R. Goldenring (2010). "Loss of Rab25 promotes the development of intestinal neoplasia in mice and is associated with human colorectal adenocarcinomas." Journal of Clinical Investigation **120**(3): 840-849.

Natoli, M., J. Christensen, S. El-Gebali, A. Felsani and P. Anderle (2013). "The role of CDX2 in Caco-2 cell differentiation." Eur J Pharm Biopharm **85**(1): 20-25.

Nawshad, A., D. Medici, C. C. Liu and E. D. Hay (2007). "TGFbeta3 inhibits E-cadherin gene expression in palate medial-edge epithelial cells through a Smad2-Smad4-LEF1 transcription complex." J Cell Sci **120**(Pt 9): 1646-1653.

Nicoletti, C. (2000). "Unsolved mysteries of intestinal M cells." Gut **47**(5): 735-739.
Nishita, M., M. K. Hashimoto, S. Ogata, M. N. Laurent, N. Ueno, H. Shibuya and K. W. Cho (2000). "Interaction between Wnt and TGF-beta signalling pathways during formation of Spemann's organizer." Nature **403**(6771): 781-785.

Nollet, F., G. Berx, F. Molemans and F. van Roy (1996). "Genomic organization of the human beta-catenin gene (CTNNB1)." Genomics **32**(3): 413-424.

Nusse, R. and H. E. Varmus (1982). "Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome." Cell **31**(1): 99-109.

Oda, K., Y. Matsuoka, A. Funahashi and H. Kitano (2005). "A comprehensive pathway map of epidermal growth factor receptor signaling." Mol Syst Biol **1**: 2005 0010.

Ohtaki, N., A. Yamaguchi, T. Goi, T. Fukaya, K. Takeuchi, K. Katayama, K. Hirose and T. Urano (2001). "Somatic alterations of the DPC4 and Madr2 genes in colorectal cancers and relationship to metastasis." Int J Oncol **18**(2): 265-270.

Olsen, A. K., M. Coskun, M. Bzorek, M. H. Kristensen, E. T. Danielsen, S. Jorgensen, J. Olsen, U. Engel, S. Holck and J. T. Troelsen (2013). "Regulation of APC and AXIN2 expression by intestinal tumor suppressor CDX2 in colon cancer cells." Carcinogenesis **34**(6): 1361-1369.

Oshima, M., H. Oshima, K. Kitagawa, M. Kobayashi, C. Itakura and M. Taketo (1995). "Loss of Apc heterozygosity and abnormal tissue building in nascent intestinal polyps in mice carrying a truncated Apc gene." Proc Natl Acad Sci U S A **92**(10): 4482-4486.

Pang, R., W. L. Law, A. C. Chu, J. T. Poon, C. S. Lam, A. K. Chow, L. Ng, L. W. Cheung, X. R. Lan, H. Y. Lan, V. P. Tan, T. C. Yau, R. T. Poon and B. C. Wong (2010). "A subpopulation of CD26+ cancer stem cells with metastatic capacity in human colorectal cancer." Cell Stem Cell **6**(6): 603-615.

Park, E. T., H. K. Oh, J. R. Gum, Jr., S. C. Crawley, S. Kakar, J. Engel, C. C. Leow, W. Q. Gao and Y. S. Kim (2006). "HATH1 expression in mucinous cancers of the colorectum and related lesions." Clin Cancer Res **12**(18): 5403-5410.

Parkin, C. A. and P. W. Ingham (2008). "The adventures of Sonic Hedgehog in development and repair. I. Hedgehog signaling in gastrointestinal development and disease." Am J Physiol Gastrointest Liver Physiol **294**(2): G363-367.

Perreault, N., B. A. Auclair, Y. D. Benoit, N. Rivard and Y. Mishina (2007). "Bone morphogenetic protein signaling is essential for terminal differentiation of the intestinal secretory cell lineage." Gastroenterology **133**(3): 887-896.

Pinchuk, I. V., R. C. Mifflin, J. I. Saada and D. W. Powell (2010). "Intestinal mesenchymal cells." Curr Gastroenterol Rep **12**(5): 310-318.

Porter, E. M., C. L. Bevins, D. Ghosh and T. Ganz (2002). "The multifaceted Paneth cell." Cell Mol Life Sci **59**(1): 156-170.

Powell, A. E., Y. Wang, Y. Li, E. J. Poulin, A. L. Means, M. K. Washington, J. N. Higginbotham, A. Juchheim, N. Prasad, S. E. Levy, Y. Guo, Y. Shyr, B. J. Aronow, K. M. Haigis, J. L. Franklin and R. J. Coffey (2012). "The pan-ErbB negative regulator Lrig1 is an intestinal stem cell marker that functions as a tumor suppressor." Cell **149**(1): 146-158.

Pretlow, T. P. and T. G. Pretlow (2005). "Mutant KRAS in aberrant crypt foci (ACF): initiation of colorectal cancer?" Biochim Biophys Acta **1756**(2): 83-96.

Radtke, F. and H. Clevers (2005). "Self-renewal and cancer of the gut: two sides of a coin." Science **307**(5717): 1904-1909.

Ramalho-Santos, M., D. A. Melton and A. P. McMahon (2000). "Hedgehog signals regulate multiple aspects of gastrointestinal development." Development **127**(12): 2763-2772.

Reinacher-Schick, A., S. E. Baldus, B. Romdhana, S. Landsberg, M. Zapatka, S. P. Monig, A. H. Holscher, H. P. Dienes, W. Schmiegel and I. Schwarte-Waldhoff (2004). "Loss of Smad4 correlates with loss of the invasion suppressor E-cadherin in advanced colorectal carcinomas." J Pathol **202**(4): 412-420.

Reya, T. and H. Clevers (2005). "Wnt signalling in stem cells and cancer." Nature **434**(7035): 843-850.

Riggins, G. J., K. W. Kinzler, B. Vogelstein and S. Thiagalingam (1997). "Frequency of Smad gene mutations in human cancers." Cancer Res **57**(13): 2578-2580.

Rijsewijk, F., M. Schuermann, E. Wagenaar, P. Parren, D. Weigel and R. Nusse (1987). "The Drosophila homolog of the mouse mammary oncogene int-1 is identical to the segment polarity gene wingless." Cell **50**(4): 649-657.

Rowan, A., S. Halford, M. Gaasenbeek, Z. Kemp, O. Sieber, E. Volikos, E. Douglas, H. Fiegler, N. Carter, I. Talbot, A. Silver and I. Tomlinson (2005). "Refining molecular analysis in the pathways of colorectal carcinogenesis." Clin Gastroenterol Hepatol **3**(11): 1115-1123.

Samuels, Y., Z. Wang, A. Bardelli, N. Silliman, J. Ptak, S. Szabo, H. Yan, A. Gazdar, S. M. Powell, G. J. Riggins, J. K. Willson, S. Markowitz, K. W. Kinzler, B. Vogelstein and V. E. Velculescu (2004). "High frequency of mutations of the PIK3CA gene in human cancers." Science **304**(5670): 554.

Sangiorgi, E. and M. R. Capecchi (2008). "Bmi1 is expressed in vivo in intestinal stem cells." Nat Genet **40**(7): 915-920.

Sato, T., J. H. van Es, H. J. Snippert, D. E. Stange, R. G. Vries, M. van den Born, N. Barker, N. F. Shroyer, M. van de Wetering and H. Clevers (2011). "Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts." Nature **469**(7330): 415-418.

Sato, T., R. G. Vries, H. J. Snippert, M. van de Wetering, N. Barker, D. E. Stange, J. H. van Es, A. Abo, P. Kujala, P. J. Peters and H. Clevers (2009). "Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche." Nature **459**(7244): 262-265.

Seshagiri, S., E. W. Stawiski, S. Durinck, Z. Modrusan, E. E. Storm, C. B. Conboy, S. Chaudhuri, Y. Guan, V. Janakiraman, B. S. Jaiswal, J. Guillory, C. Ha, G. J. Dijkgraaf, J. Stinson, F. Gnad, M. A. Huntley, J. D. Degenhardt, P. M. Haverty, R. Bourgon, W. Wang, H. Koeppen, R. Gentleman, T. K. Starr, Z. Zhang, D. A. Largaespada, T. D. Wu and F. J. de Sauvage (2012). "Recurrent R-spondin fusions in colon cancer." Nature **488**(7413): 660-664.

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

Shim, J. H., C. Xiao, A. E. Paschal, S. T. Bailey, P. Rao, M. S. Hayden, K. Y. Lee, C. Bussey, M. Steckel, N. Tanaka, G. Yamada, S. Akira, K. Matsumoto and S. Ghosh (2005). "TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo." Genes Dev **19**(22): 2668-2681.

Shiou, S. R., A. B. Singh, K. Moorthy, P. K. Datta, M. K. Washington, R. D. Beauchamp and P. Dhawan (2007). "Smad4 regulates claudin-1 expression in a transforming growth factor-beta-independent manner in colon cancer cells." Cancer Res **67**(4): 1571-1579.

Shroyer, N. F., M. A. Helmrath, V. Y. Wang, B. Antalffy, S. J. Henning and H. Y. Zoghbi (2007). "Intestine-specific ablation of mouse atonal homolog 1 (Math1) reveals a role in cellular homeostasis." Gastroenterology **132**(7): 2478-2488.

Shtutman, M., J. Zhurinsky, I. Simcha, C. Albanese, M. D'Amico, R. Pestell and A. Ben-Ze'ev (1999). "The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway." Proceedings of the National Academy of Sciences of the United States of America **96**(10): 5522-5527.

Siegel, R., D. Naishadham and A. Jemal (2013). "Cancer statistics, 2013." CA Cancer J Clin **63**(1): 11-30.

Smith, J. J., N. G. Deane, F. Wu, N. B. Merchant, B. Zhang, A. Jiang, P. Lu, J. C. Johnson, C. Schmidt, C. E. Bailey, S. Eschrich, C. Kis, S. Levy, M. K. Washington, M. J. Heslin, R. J. Coffey, T. J. Yeatman, Y. Shyr and R. D. Beauchamp (2010).

"Experimentally derived metastasis gene expression profile predicts recurrence and death in patients with colon cancer." Gastroenterology **138**(3): 958-968.

Smyth, G. K. (2004). "Linear models and empirical bayes methods for assessing differential expression in microarray experiments." Stat Appl Genet Mol Biol **3**: Article3.
Specian, R. D. and M. G. Oliver (1991). "Functional biology of intestinal goblet cells." Am J Physiol **260**(2 Pt 1): C183-193.

Stelzner, M., M. Helmrath, J. C. Dunn, S. J. Henning, C. W. Houchen, C. Kuo, J. Lynch, L. Li, S. T. Magness, M. G. Martin, M. H. Wong, J. Yu and N. I. H. I. S. C. Consortium (2012). "A nomenclature for intestinal in vitro cultures." Am J Physiol Gastrointest Liver Physiol **302**(12): G1359-1363.

Sun, L., G. Wu, J. K. Willson, E. Zborowska, J. Yang, I. Rajkarunanayake, J. Wang, L. E. Gentry, X. F. Wang and M. G. Brattain (1994). "Expression of transforming growth factor beta type II receptor leads to reduced malignancy in human breast cancer MCF-7 cells." J Biol Chem **269**(42): 26449-26455.

Tachezy, M., H. Zander, F. Gebauer, A. Marx, J. T. Kaifi, J. R. Izbicki and M. Bockhorn (2012). "Activated leukocyte cell adhesion molecule (CD166)--its prognostic power for colorectal cancer patients." J Surg Res **177**(1): e15-20.

Takaku, K., M. Oshima, H. Miyoshi, M. Matsui, M. F. Seldin and M. M. Taketo (1998). "Intestinal tumorigenesis in compound mutant mice of both Dpc4 (Smad4) and Apc genes." Cell **92**(5): 645-656.

Takeyama, K., K. Dabbagh, H. M. Lee, C. Agusti, J. A. Lausier, I. F. Ueki, K. M. Grattan and J. A. Nadel (1999). "Epidermal growth factor system regulates mucin production in airways." Proc Natl Acad Sci U S A **96**(6): 3081-3086.

Tanaka, T., T. Watanabe, Y. Kazama, J. Tanaka, T. Kanazawa, S. Kazama and H. Nagawa (2008). "Loss of Smad4 protein expression and 18qLOH as molecular markers indicating lymph node metastasis in colorectal cancer--a study matched for tumor depth and pathology." J Surg Oncol **97**(1): 69-73.

ten Dijke, P. and O. Korchynskyi (2002). "Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter." Journal of Biological Chemistry **277**(7): 4883-4891.

Thiagalingam, S., C. Lengauer, F. S. Leach, M. Schutte, S. A. Hahn, J. Overhauser, J. K. Willson, S. Markowitz, S. R. Hamilton, S. E. Kern, K. W. Kinzler and B. Vogelstein (1996). "Evaluation of candidate tumour suppressor genes on chromosome 18 in colorectal cancers." Nat Genet **13**(3): 343-346.

Thibodeau, S. N., G. Bren and D. Schaid (1993). "Microsatellite instability in cancer of the proximal colon." Science **260**(5109): 816-819.

Tian, H., B. Biehs, S. Warming, K. G. Leong, L. Rangell, O. D. Klein and F. J. de Sauvage (2011). "A reserve stem cell population in small intestine renders Lgr5-positive cells dispensable." Nature **478**(7368): 255-259.

Tian, X., H. Du, X. Fu, K. Li, A. Li and Y. Zhang (2009). "Smad4 restoration leads to a suppression of Wnt/beta-catenin signaling activity and migration capacity in human colon carcinoma cells." Biochem Biophys Res Commun **380**(3): 478-483.

Todaro, M., M. G. Francipane, J. P. Medema and G. Stassi (2010). "Colon cancer stem cells: promise of targeted therapy." Gastroenterology **138**(6): 2151-2162.

van de Wetering, M., E. Sancho, C. Verweij, W. de Lau, I. Oving, A. Hurlstone, K. van der Horn, E. Batlle, D. Coudreuse, A. P. Haramis, M. Tjon-Pon-Fong, P. Moerer, M. van den Born, G. Soete, S. Pals, M. Eilers, R. Medema and H. Clevers (2002). "The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells." Cell **111**(2): 241-250.

van den Brink, G. R., S. A. Bleuming, J. C. Hardwick, B. L. Schepman, G. J. Offerhaus, J. J. Keller, C. Nielsen, W. Gaffield, S. J. van Deventer, D. J. Roberts and M. P. Peppelenbosch (2004). "Indian Hedgehog is an antagonist of Wnt signaling in colonic epithelial cell differentiation." Nat Genet **36**(3): 277-282.

van Dop, W. A., J. Heijmans, N. V. Buller, S. A. Snoek, S. L. Rosekrans, E. A. Wassenberg, M. A. van den Bergh Weerman, B. Lanske, A. R. Clarke, D. J. Winton, M. Wijgerde, G. J. Offerhaus, D. W. Hommes, J. C. Hardwick, W. J. de Jonge, I. Biemond and G. R. van den Brink (2010). "Loss of Indian Hedgehog activates multiple aspects of

a wound healing response in the mouse intestine." Gastroenterology **139**(5): 1665-1676, 1676 e1661-1610.

van Es, J. H., M. E. van Gijn, O. Riccio, M. van den Born, M. Vooijs, H. Begthel, M. Cozijnsen, S. Robine, D. J. Winton, F. Radtke and H. Clevers (2005). "Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells." Nature **435**(7044): 959-963.

VanDussen, K. L., A. J. Carulli, T. M. Keeley, S. R. Patel, B. J. Puthoff, S. T. Magness, I. T. Tran, I. Maillard, C. Siebel, A. Kolterud, A. S. Grosse, D. L. Gumucio, S. A. Ernst, Y. H. Tsai, P. J. Dempsey and L. C. Samuelson (2012). "Notch signaling modulates proliferation and differentiation of intestinal crypt base columnar stem cells." Development **139**(3): 488-497.

VanDussen, K. L. and L. C. Samuelson (2010). "Mouse atonal homolog 1 directs intestinal progenitors to secretory cell rather than absorptive cell fate." Dev Biol **346**(2): 215-223.

Vazquez, A., E. E. Bond, A. J. Levine and G. L. Bond (2008). "The genetics of the p53 pathway, apoptosis and cancer therapy." Nat Rev Drug Discov **7**(12): 979-987.

Vermeulen, L., E. M. F. De Sousa, M. van der Heijden, K. Cameron, J. H. de Jong, T. Borovski, J. B. Tuynman, M. Todaro, C. Merz, H. Rodermond, M. R. Sprick, K. Kemper, D. J. Richel, G. Stassi and J. P. Medema "Wnt activity defines colon cancer stem cells and is regulated by the microenvironment." Nat Cell Biol **12**(5): 468-476.

Vogelstein, B., E. R. Fearon, S. R. Hamilton, S. E. Kern, A. C. Preisinger, M. Leppert, Y. Nakamura, R. White, A. M. Smits and J. L. Bos (1988). "Genetic alterations during colorectal-tumor development." N Engl J Med **319**(9): 525-532.

Vousden, K. H. and C. Prives (2009). "Blinded by the Light: The Growing Complexity of p53." Cell **137**(3): 413-431.

Wang, L. C., F. Nassir, Z. Y. Liu, L. Ling, F. Kuo, T. Crowell, D. Olson, N. O. Davidson and L. C. Burkly (2002). "Disruption of hedgehog signaling reveals a novel role in intestinal morphogenesis and intestinal-specific lipid metabolism in mice." Gastroenterology **122**(2): 469-482.

Weichert, W., T. Knosel, J. Bellach, M. Dietel and G. Kristiansen (2004). "ALCAM/CD166 is overexpressed in colorectal carcinoma and correlates with shortened patient survival." J Clin Pathol **57**(11): 1160-1164.

Willert, K., J. D. Brown, E. Danenberg, A. W. Duncan, I. L. Weissman, T. Reya, J. R. Yates, 3rd and R. Nusse (2003). "Wnt proteins are lipid-modified and can act as stem cell growth factors." Nature **423**(6938): 448-452.

Winesett, M. P., G. W. Ramsey and J. A. Barnard (1996). "Type II TGF(beta) receptor expression in intestinal cell lines and in the intestinal tract." Carcinogenesis **17**(5): 989-995.

Woodford-Richens, K. L., A. J. Rowan, P. Gorman, S. Halford, D. C. Bicknell, H. S. Wasan, R. R. Roylance, W. F. Bodmer and I. P. Tomlinson (2001). "SMAD4 mutations in colorectal cancer probably occur before chromosomal instability, but after divergence of the microsatellite instability pathway." Proc Natl Acad Sci U S A **98**(17): 9719-9723.

Yamashita, M., K. Fatyol, C. Jin, X. Wang, Z. Liu and Y. E. Zhang (2008). "TRAF6 mediates Smad-independent activation of JNK and p38 by TGF-beta." Mol Cell **31**(6): 918-924.

Yang, L., L. Wang and X. Yang (2009). "Disruption of Smad4 in mouse epidermis leads to depletion of follicle stem cells." Mol Biol Cell **20**(3): 882-890.

Yang, Q., N. A. Bermingham, M. J. Finegold and H. Y. Zoghbi (2001). "Requirement of Math1 for secretory cell lineage commitment in the mouse intestine." Science **294**(5549): 2155-2158.

Yang, Y. Y., P. C. Lee, Y. T. Huang, W. P. Lee, Y. J. Kuo, K. C. Lee, Y. C. Hsieh, T. Y. Lee and H. C. Lin (2014). "Involvement of the HIF-1alpha and Wnt/beta-catenin pathways in the protective effects of losartan on fatty liver graft with ischaemia/reperfusion injury." Clin Sci (Lond) **126**(2): 163-174.

Yu, L., M. C. Hebert and Y. E. Zhang (2002). "TGF-beta receptor-activated p38 MAP kinase mediates Smad-independent TGF-beta responses." EMBO J **21**(14): 3749-3759.
Yue, J. and K. M. Mulder (2000). "Activation of the mitogen-activated protein kinase pathway by transforming growth factor-beta." Methods Mol Biol **142**: 125-131.

Zhang, T., L. B. Nanney, M. O. Peeler, C. S. Williams, L. Lamps, K. J. Heppner, R. N. DuBois and R. D. Beauchamp (1997). "Decreased transforming growth factor beta type II receptor expression in intestinal adenomas from Min/+ mice is associated with increased cyclin D1 and cyclin-dependent kinase 4 expression." Cancer Res **57**(9): 1638-1643.

Zhang, Y., X. Feng, R. We and R. Derynck (1996). "Receptor-associated Mad homologues synergize as effectors of the TGF-beta response." Nature **383**(6596): 168-172.

Zhang, Y. E. (2009). "Non-Smad pathways in TGF-beta signaling." Cell Res **19**(1): 128-139.