**UNIVERSITY OF CALIFORNIA, SAN DIEGO**

# The Role of Bone Morphogenetic Protein in Colorectal Cancer

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

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2006

The dissertation of Stayce Beck is approved, and it is acceptable in quality and form for publication on microfilm:

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Chair

University of California, San Diego

2006

## DEDICATION

To my parents, for all of their support and always believing in me.

## TABLE OF CONTENTS

## LIST OF FIGURES

### Chapter 1



### Chapter 3

**Chapter 4**

**Chapter 5**

**Chapter 6**

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# Curriculum Vitae

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**Abstracts:**

1. Fiorino A, Grady WM, Jung B, Cabrera B, Munoz N, **Beck S**, O'Keefe M, Carethers JM "Correction of microsatellite instability and restoration of Transforming Growth Factor –β pathway induces growth suppression in HCT116+chr. 3 colon cancer cells through c-MYC downregulation and $p21^{Cip/WAF1}$ upregulation". Proc. Am. Assoc. Cancer Res., #5964, 2003.

2. **Beck S**, Fiorino A, Cabrera B, Jung B, Carethers J. BMP signaling is intact but not significantly growth suppressive in colon and endometrial cancer cells. Proc. Am. Assoc. Cancer Res., #2741, 2004.

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8. **Beck S**, Jung B, Del Rosario E, Gomez J, and Carethers J "Activated RAS modulates Bone Morphogenetic Protein (BMP)-induced Growth Suppression." AACR Special Conferences in TGF-β San Diego, CA Abstract B35, 2006

9. **Beck S**, Jung B, Del Rosario E, Gomez J, and Carethers J. "Modulation of Bone Morphogenetic Protein (BMP)-induced Growth Suppression by Activated RAS." Gastroenterology 130 (4): A226640 Suppl. 2, APR 2006

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2. **Beck SE**, Jung B, Del Rosario E, Gomez J, and Carethers J. "Activated RAS modulates Bone Morphogenetic Protein (BMP)-induced Growth Suppression." *(In Submission)*

3. Jung B, **Beck SE**, Cabrera B, Fiorino A, Smith EJ, Bocanegra M, and Carethers J. "Activin type 2 receptor (ACVR2) restoration in microsatellite unstable colon cancer leads to growth suppression and enhanced migration following activin treatment." *(In Submission*)

4. **Beck SE**, Sabates J, Carter J, and Carethers J. "Bone Morphogenetic Protein Exerts a SMAD4 Independent Effect in Colon Cancer Cells." *(In Submission)*

5. Fiorino A, **Beck SE**, Cabrera B, Furnari F, and John M. Carethers. "Phosphatidylinositol 3-kinase activity and RAS/MAPK/ERK cascade interferes with the TGFb-SMAD signaling by distinct mechanisms in responsive HCT116+chr3 and FET colon cancer cells." *(In Submission)*

6. Jimmy Y.C. Chow JYC, Cabral J, Cabrera BL, **Beck SE**, and Carethers JM. "RAS/ERK Modulates TGFβ-regulated *PTEN* Expression In Human Pancreatic Adenocarcinoma Cells" *(In Submission)*

## ABSTRACT OF THE DISSERTATION

### The Role of Bone Morphogenetic Protein in Colorectal Cancer

by

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Normally growth suppressive in epithelial cells, the transforming growth factor beta superfamily has been shown to be disrupted in many types of cancers. Bone Morphogenetic Protein (BMP), a member of the transforming growth factor beta superfamily has been found to be intact and growth suppressive in a variety of cancers, but it has been found to be targeted for mutation in the colon cancer predisposition syndrome Juvenile Polyposis, and little is known about its role in colon cancer. In this dissertation, we attempt to understand what role the BMP signaling pathway plays in colon cancer. We aimed to do this by: a) determining if the BMP pathway is intact in microsatellite stable and unstable colon cancer cell lines and tissues, b) determining if the Ras pathway affects BMP signaling, and c) examining SMAD4-independent means of BMP signaling. We found that while the BMP pathway was intact in many of the colon cancer cell lines examined, the RAS pathway interfered with transmission of SMAD signaling to the nucleus. BMP2 was

shown to increase the stability of p21, and the RAS/ERK pathway acts to dull the growth suppressiveness and decrease the stability of p21. Additionally, we found that in the absence of SMAD4, which is targeted for mutation in Juvenile Polyposis and some types of colon cancers, that BMP treatment becomes growth proliferative over time by decreasing cellular levels of PTEN through the RAS/ERK pathway. Our results suggest that while the BMP pathway is often intact in colon cancer cell lines, its effects are modulated by other signaling pathways.

# Chapter 1:

# Introduction

**Transforming Growth Factor Beta Superfamily Introduction**

Bone Morphogenetic Proteins (BMP) are members of the Transforming Growth Factor-β superfamily (TGFβ) that induce bone formation and affect proliferation and differentiation of certain osteoblastic cells (Wozney et al., 1988; Yamaguchi et al., 1991). The TGFβ superfamily is composed of extra-cellular growth factors and include: TGFβ, activin, the BMPs, growth differentiation factors (GDFs), inhibins, Müllerian inhibiting substance (MIS), Nodal, and Leftys. These ligands are translated as prepropeptide precursors, which are processed and secreted as homo- and hetero-dimers (Chang et al., 2002). Members of the TGFβ pathway are intimately involved in the development process and defects in the ligands, receptors, and downstream signaling mediators can result in many developmental defects. Experiments involving knock out mice have shown that these growth factors play a role in: heart development, left-right symmetry, vasculogenesis and angiogenesis, craniofacial development, skeletal morphogenesis, body composition, nervous system development, sexual differentiation, and primordial germ cell and gonadal development (Aubin et al., 2004; Chang et al., 2002).

Members of the TGFβ family exert their signaling effect to the cell by binding to type I and II membrane receptors (Koenig et al., 1994; ten Dijke et al., 1994a; ten Dijke et al., 1994b; ten Dijke et al., 1994c; Yamashita et al., 1995). In particular, there are 3 type II receptors and six type I receptors. TGFβ

predominately utilizes the TGFβ type I and II receptors (TGFβRI and TGβRII, respectively) by binding to first the type II receptor and then recruiting the type I receptor. Activin utilizes a similar binding pattern by binding to the Activin type I and II receptors (ACVRI and ACVRII, respectively), while BMPs utilize BMP type I and II receptors (BMPRI and BMPRII, respectively). Ligand binding and dimerization of the receptors induces phosphorylation of the type I receptor by the type II receptor. Intracellular *mothers against decapentaplegic, Drosophila* (SMADs) are then recruited to the receptor and are phosphorylated by the type I receptor. TGFβ and activin utilize SMAD2 and SMAD3, while BMPs utilize SMADs 1, 5 or 8. The phosphorylated SMADs then bind with the co-SMAD, SMAD4, and together these translocate to the nucleus to turn on transcription of target genes (Kretzschmar et al., 1997b; Liu et al., 1996; Liu et al., 1995; Nohe et al., 2003).

There are approximately 19 mammalian BMPs, all of which contain seven highly conserved cysteines in their carboxyl-terminal portions that identifies them as being members of the TGFβ superfamily (Derner & Anderson, 2005). In 1991, Yamaguchi et al. discovered that recombinant BMP2 had differential effects on osteoblasts at varying stages of differentiation. BMP2 stimulates the growth of osteoprogenitor C26 cells, which can differentiate into muscle cells and adipocytes, while C20 cells, which are more differentiated osteoblast like cells, are slightly inhibited by BMP2 addition (Yamaguchi et al., 1991). Yamaguchi et al. also

determined that BMP2 stimulates differentiation of mesenchymal cells into osteoblastic cells and inhibits myogenic differentiation of these cells to muscle cells (Yamaguchi et al., 1991; Yamamoto et al., 1997). In 1992, Paralkar et al. discovered BMP4 can induce the differentiation of PC12 cells and that extracellular matrix molecules, type IV collagen in particular, can potentiate this effect (Paralkar et al., 1992). While TGFβ and BMP are from the same family and both inhibit myogenic differentiation, they have different actions on osteoblast differentiation and their mRNAs are expressed in different populations of mesenchymal cells in the developing skeletal system (Lyons et al., 1989).

**BMP Receptors**

The 19 known BMP ligands, have been shown to activate only three BMP-type-I receptors and three BMP-type-II receptors to mediate the responses of these ligands. BMP receptor type IA (BMPRIA) is located at chromosome 10q22.3 and BMP receptor type IB (BMPRIB) is located at 4q23-q24, while BMPRII is assigned to 2q33-34 and a processed BMPRIA pseudogene was mapped to 6q23 (Anna-Karin et al., 1999; Idea BF, 1998). Therefore, to exert their diverse effects on cells, BMP receptors have a much more flexible oligomerization pattern than that of TGFβ and activin. BMP receptors exist at the cell surface in the absence of ligand as pre-formed heterodimers of a type I and type II receptor, or as homomeric oligomers, whereas TGFβ receptors are believed to be fully homomeric in the

absence of ligand. This novel feature in the oligomerization mode of the BMP receptors allows for more variety in the response to various ligands (Gilboa et al., 2000). Nohe et al. found that BMP-2 stimulation of cells leads to a rearrangement of receptor complexes at the cell surface and BMPRII is necessary for this rearrangement (Nohe et al., 2003). Additionally, BMP's utilize a slightly different binding pattern than the traditional TGFβ and activin ligands. BMP either binds concomitantly to the type I and II receptors, or it binds first to the type I receptor, and then recruits the type II receptor (Nohe et al., 2002), while TGFβ and activin bind first to the type II receptor and then recruit the type I receptor (Attisano et al., 1993; Wrana et al., 1992).

BMP's bind to several type I and II receptors according to the affinity that the different BMP's have for the receptors, and the presence of the receptors, further increasing the diversity of the signal BMPs can transmit to the cell. Predominantly, BMP2 and BMP4 bind to BMPRIA and BMPRII, while BMP7 prefers to bind to BMPRIA and ACVRII (Macias-Silva et al., 1998; Murakami et al., 2003; Nickel et al., 2001; Sebald & Mueller, 2003).

SMAD recognition by the type I receptor is determined by two structural elements: the L3 loop in the carboxy-terminus (MH2) of the SMAD and the L45 loop in the kinase domain of the receptor (Chen et al., 1998). Each of these elements contain short amino acid sequences that are highly conserved between SMAD proteins or receptor, but differ in a few residues between the distinct

SMADs or receptor. The L45 loop on the TGFβ/activin type I receptors corresponds with the L3 loop sequence common to SMAD2/SMAD3, which allows for functional interactions between them. Similarly, the L45 loop on the BMPRI group corresponds to the L3 loop of SMAD1, 5 and 8, conferring specificity for the interactions between these receptors and SMAD proteins. Interestingly, activin like kinase 2 (Alk2), the BMP7 preferred type I receptor is able to phosphorylate and activate Smad1 even though the L45 sequence of this group is very divergent from that of BMPRI. This is due to the recognition of an α-helix1 in the L3 loop of SMAD1 on the surface of the MH2 domain (Chen & Massague, 1999; Murakami et al., 2003; Scheufler et al., 1999).

**Intracellular SMAD Proteins**

SMAD proteins play pivotal roles in the intracellular signaling of the multifunctional TGF-β family members downstream of serine/threonine kinase type I and type II receptors. There are eight mammalian SMADs, and six of these have a conserved N-terminal MH1 domain and a C-terminal MH2 domain, which are linked by a proline-rich linker region that varies in length and sequence between the SMADs. The MH2 domain is believed to act as the effecter domain, while the MH1 domain negatively regulates the MH2 domain (Baker & Harland, 1996; Hata et al., 1997). SMADs 1, 2, 3, 5,and 8 contain a Ser-Ser-X-Ser (SSXS) region that becomes directly phosphorylated by the type I receptors at the last two

serines (**Figure 1.1**) (Macias-Silva et al., 1996). SMAD6 and SMAD7 are known as inhibitory SMADs because they inhibit the other SMADs, and while they have MH2 domains, they do not contain SSXS phosphorylation motifs and do not have conserved N-terminal domains. SMAD2 and SMAD3 are specific mediators of TGF-β and activin responses, while SMAD1, SMAD5 and SMAD8 are involved in BMP signaling (Hoodless et al., 1996; Kiyoshi Tamaki, 1998; Kretzschmar et al., 1997b; Tamaki K, 1998; Zhang Y, 1996). An important avenue of research will be to decipher the varying roles of the three BMP triggered SMADs in the cell and what upstream signals dictate which SMAD gets activated. Once phosphorylated, the SMADs are released from the receptor and bind to SMAD4, known as the co-SMAD (Lagna et al., 1996; Zhang et al., 1997). SMAD4 itself does not bind to the receptors, but forms a homo-oligomer and when the other SMADs bind, it is believed it can become a hetero-hexamer, with heter-oligomers of SMAD2/SMAD3 or SMAD1/5/8. (Lagna G, 1996; Shi et al., 1997) After heteroligomerization, the SMADs translocate to the nucleus and act as transcription factors (**Figure 1.2**). The TGFβ superfamily members transactivate a variety of genes including: PAI-1(Keeton et al., 1991) and the cyclin dependent kinase inhibitors p15 and p21 for TGFβ (Datto et al., 1995; Li et al., 1995). Id2 and collagen-X are transactivated following BMP signaling (Izumi et al., 2005; Katerina Pardali, 2005; Pardali K, 2005; Yamashita et al., 1995).

**Regulation of BMP Signaling**

The bone morphogenetic proteins are potent regulators of cell growth and differentiation in mammals. Correspondingly, there is a complex series of regulators that control multiple levels of the BMP signaling cascade including: direct binding or regulators to the BMPs, preventing them from binding to the receptors; regulators acting as direct SMAD inhibitors by binding to the SMADs; and regulators of SMAD transcription inhibitors (Di Chen, 2004). The secreted BMP antagonists include noggin, gremlin, DAN and chordin. Noggin, a member of the cysteine knot family of proteins, is an extracellular protein that binds to BMP and prevents it from binding to the receptors (Groppe et al., 2002; Groppe et al., 2003). The noggin homo-dimer binds to the BMP homo-dimer at both the type I receptor binding interface using the N-terminal half of noggin and the type II receptor interface via the C-terminal half of noggin, thus blocking BMP from binding to the receptor (Groppe et al., 2003). Gremlin and DAN are homologous antagonists that also contain the conserved cysteine knot domain shared by the TGFβ superfamily that hetero-dimerize with BMP2, 4 and 7 preventing them from binding to their receptors (Pearce et al., 1999; Topol et al., 2000). Chordin contains four cysteine-rich domains that bind to BMP in the extracellular space preventing it from receptor binding (Hyvonen, 2003; Larrain et al., 2000). BMP signaling to the nucleus is also inhibited through SMAD interactions. SMAD6 and SMAD7 are designated inhibitory SMADs because they bind to the receptor SMADs (SMAD1,

2, 3, 5, and 8). SMAD7 blocks both BMP and TGFβ signaling. SMAD6 competes with SMAD4 for binding to receptor-phosphorylated SMAD1, generating an inactive SMAD1-SMAD6 complex (Hata et al., 1998; Imamura et al., 1997). It has also been shown that SMAD6 interacts with homeobox c-8 acting as a transcriptional co-repressor, which inhibits the transcriptional activity of SMAD1 acting as a negative feedback loop of BMP signaling (Bai et al., 2000). Ski and SnoN are other important negative regulators of TGFβ superfamily signaling. These proteins act by binding to BMP-SMAD complexes and disrupting their ability to activate BMP target genes.

Ski and SnoN interact with SMAD2, 3, and 4 and are recruited to the SMAD binding element in the TGFβ responsive promoters, repressing the ability of SMADs to activate their TGFβ target genes (Akiyoshi et al., 1999; Luo et al., 1999). Ski is also able to interact with the SMAD4/SMAD1 or SMAD5 complexes, blocking BMP transcriptional activation of target genes (Luo, 2003; Wang et al., 2000). Another inhibitor of BMP-induced SMAD transcription that acts by associating with SMAD1 and 5 is Tob (Yoshida et al., 2000; Yoshida et al., 2003).

Yet another inhibitory protein targets the SMAD proteins for degradation, Smad ubiquitin regulatory factor (Smurf) 1, mediates degradation of Smad1 and 5 by directly interacting with them, as well as by indirectly interacting with them by binding to the inhibitory SMADs. Additionally, Smurf1 can target the BMP type I receptors for degradation by binding to SMAD6 (Murakami et al., 2003).

**RAS Signaling**

Ras proteins are small GTP-binding proteins that activate many signaling cascades which mainly regulate gene expression (Boguski & McCormick, 1993; Takai et al., 2001). Ras switches between an active and inactive state by being bound to GTP (active) and bound to GDP (inactive) in response to upstream signals including epidermal growth factor (EGF) (Boguski & McCormick, 1993). Guanine nucleotide exchange factors (GEF) catalyze the exchange of bound GDP to cellular GTP, while a GTPase activating protein (GAP) induces the hydrolysis of bound GTP reverting back to GDP and making Ras inactive (Takai et al., 2001). One Ras activating signal is EGF, and when EGF binds to the EGF Receptor (EGFR), the receptor dimerizes and autophosphorylates tyrosine residues in its cytoplasmic domain which allows for Src- Homology Domains (SH2) domains on other proteins to bind (Bruce Alberts, 2002). SH2 domains are approximately 100 amino acids long and have a dynamic phosphotyrosine-binding motif, and act as adapters of signaling proteins to receptors as well as adaptors of multiple cytoplasmic signaling proteins bringing different proteins in close proximity with each other and allowing them to interact (Bruce Alberts, 2002). Growth factor receptor binding protein (Grb2) is one adaptor protein that binds to the GEF, son of sevenless (SOS1) via its Src Homology 3 (SH3) domain (Boguski & McCormick, 1993). An SH3 domain is approximately 50 amino acids long and contains a static polyproline binding domain (Bruce Alberts, 2002). Cytoplasmic Sos1 is brought to the plasma

membrane inducing Ras activation. Additionally there are various other RasGEFs including cdc25, Bud5, Ras-GRF, RAS-GRP, Lte1, and Scd25 that activate Ras in response to other signals (Boguski & McCormick, 1993). Important GAPs include p120GAP and neurofibromin, which is the gene product of a tumor suppressor gene and mutated in von Recklinghausen neurofibromatosis (a benign tumor) (Boguski & McCormick, 1993; Takai et al., 2001).

Ras proteins are 21 kDa proto-oncogenes involved in a variety of signal transduction pathways leading to control of cellular proliferation, differentiation and death. There three main Ras's, Ha-Ras (HRAS), Ki-Ras (KRAS), and N-Ras, which are capable of transforming mammalian cells when activated by point mutations (Takai et al., 2001). Approximately 30% of human tumors have a hyperactive Ras mutation due to a point mutation at amino codon 12, 13 or 61 hindering them from the effects of GAPs and keeping them locked in the active GTP-bound state allowing them to continually promote growth (Bruce Alberts, 2002). Activated Ras exerts its effects on multiple downstream effecter molecules, one of which is Raf (Morrison et al., 1993), a serine-threonine kinase that activates MEK1 and 2 kinases, which then activate Erk kinases 1 and 2 (Blank et al., 1996) (**Figure 1.3**). Once phosphorylated at both a threonine and a tyrosine, ERK 1 and 2 are free to phsosphorylate other cytoplasmic targets as well as translocate to the nucleus and stimulate the activity of assorted transcription factors that generally promote cell growth.

Ras is also able to activate phosphatidylinositol 3-kinase (PI3 kinase), which results in the activation of Akt/PKB which controls the activity of Rac and $p70^{S6K}$ when activated by receptor tyrosine kinases (KauffmanZeh et al., 1997; Rodriguez-Viciana et al., 1994). PI3 kinase principally phosphorylates inositol phospholipids and is activated by receptor tyrosine kinases, integrins, B- and T-cell receptors, cytokine receptors, G-protein coupled receptors, and other stimuli. Activated PI3 kinase catalyzes the phosphorylation of phosphatidylinositol(3,4) phosphate 2 (PIP2) to phosphatidylinositol (3,4,5) triphosphates (PIP3), which then acts as a dock for proteins through their pleckstrin homology (PH) domains, bringing them closer together. PI3 kinase indirectly activates protein kinase B (Akt) at the cell surface allowing phophoinositol dependent kinase 1 (PDK1) to phosphorylate Akt (Bruce Alberts, 2002) (**Figure 1.4**). The three Akt isoforms (Akt1, Akt2 and Akt3) then act as kinases in the cytoplasm, phosphorylating and inhibiting many target proteins that regulate cell death (BAD), as well as acting as regulators of insulin signaling and glucose metabolism (Hennessy et al., 2005). Additionally, Akts have an effect on cell growth through activation of mTOR and $p70^{S6}$ kinase pathways (Polunovsky et al., 2000; Wendel et al., 2004), as well as cell cycle and cell proliferation through its direct action on the CDK inhibitors p21 and p27 (Shin et al., 2002; Zhou et al., 2001a), and its indirect effect on the levels of cyclin D1 and p53 through mdm2 (Hennessy et al., 2005). PI3K facilitates oncogenic β-catenin, a key oncoprotein involved in the initial stages of colonic neoplasia. This in turn

allows GSK-3β to maintain an inhibitory phosphorylation, preventing its association with the tumor suppressor APC, which allows β-catenin to translocate to the nucleus and act as a transcription factor.

Phosphatase and tensin homolog (PTEN) is a phosphatase that dephosphorylates PIP3 to PIP2, thereby counteracting the actions of PI3 kinase, leading to GSK-3β/APC association and subsequent degradation of β-catenin in the cytoplasm. When PTEN is mutated such that it is inactive, or APC is mutated, β -catenin will not be degraded and will accumulate in the nucleus where it increases growth and proliferation of the cell by inducing the expression of c-myc, c-fos, c-jun, cyclin D1 and cyclin D3 (Fiorino et al., 2001). There is evidence suggesting that PTEN may provide an alternative mechanism to disrupt gatekeeper functions in enterocytes, potentially leading to tumor progression (Dicuonzo et al., 2001).

**BMP signaling crosstalk with other signaling pathways**

Integration of diverse signaling pathways is essential in development and homeostasis for cells to interpret context-dependent cues. It has been shown that both BMP and MAPK signaling pathways converge on Smads, resulting in differential phosphorylation. Kretzschmar et al. found that ERK can phosphorylate SMAD1 at the linker region in response to EGF, in contrast to phosphorylation at the C-terminal region by BMPRI in response to BMP ligand, and that this linker phosphorylation inhibits phospho-SMAD1 from translocating to the nucleus

(Aubin et al., 2004; Kretzschmar et al., 1997a; Kretzschmar et al., 1997b). Additionally, Hu et al. found that BMP7 exerts dose-dependent stimulatory and inhibitory effects during renal branching morphogenesis by activating the p38 kinase pathway (Hu et al., 2004). Lou et al. was the first group to show that BMP2 can stimulate Erk production and activity by examining their interaction in a mesenchymal progenitor cell line C3H10T1/2. They found that BMP2 treatment upregulated Erk1 and Erk2 at the mRNA level, and induced Erk phosphorylation (Lou et al., 2000). Cells respond to a variety of signals and have to integrate many signals to respond properly. Previous work by the above mentioned groups suggests some level of interaction between the BMP signaling pathway and the RAS/MAPK signaling pathways, but further examination of these interactions is needed to fully elucidate the mechanisms and importance of these interactions.

**BMPs and GI Development**

BMPs are known to play an important role in the morphogenesis of various tissues and organs including the gastrointestinal (GI) tract in vertebrates (BL, 1996). The colon consists of three germ layers, the mesoderm, which forms the smooth muscle layer, the endoderm making up the epithelial lining and the ectoderm, which includes the enteric nervous system. De Santa Barbara et al. showed that the BMP signaling pathway is activated in all three tissues of the developing chick gut using immunohistochemistry and staining for phospho-

SMAD1, suggesting that endogenous BMP activity is present and necessary for normal gut development and differentiation (De Santa Barbara P, 2005). Haramis et al. showed that BMP4 is expressed in the intravillus mesenchyme and that villus epithelial cells respond to BMP signaling. Haramis et al. went on to show that transient noggin expression induced the formation of ectopic crypt units similar in histopathology to juvenile polyposis (Haramis et al., 2004). BMPRIA conditional knockout mice also develop polyps resembling juvenile polyps (Drucilla, 2000; Mishina Y, 2002; Yuji Mishina, 2002). Villi are tube-shaped epithelial exvaginations that line the small intestine and are rapidly turned over. Intestinal stem cells sit at the base of the crypt and as the progeny cells differentiate they move up the crypt-villus axis. When progeny enter the base of the villi, they are mature and able to carry out their main function to absorb nutrients (Haramis et al., 2004; Radtke & Clevers, 2005). In October 2004, He et al. found evidence that BMP regulates the PTEN/Akt pathway in intestinal stem cells (ISC's) using conditional BMPRIA knockout mice. They found BMP has a role in inhibiting ISC self-renewal by increasing PTEN activity and inhibiting nuclear β-catenin. They also found that β-catenin activity can be regulated by BMP through BMPRIA and PI3Kinase/Akt. They believe there is a second signal involved, such as transient expression of Noggin (a BMP specific inhibitor) to override the effects of BMP, releasing the inhibition of β-catenin by PTEN to coordinate with Wnt signaling to activate stem cells (He et al., 2004).

**BMP and Cancer**

Stem cells are able to proliferate by the symmetrical division of cellular contents and to form specialized cell types through asymmetrical cell division (Chambers I, 2004). Stem cells share many of the characteristics of cancer cells including the ability to proliferate by a process of self-renewal and a loss of contact inhibition (Gammill & Bronner-Fraser, 2002). It has been shown in a variety of systems that BMPs play key roles in determining stem cell fate during differentiation (Gammill & Bronner-Fraser, 2002; Shah et al., 1996; Ying et al., 2003). Because stem cells are primarily responsible for the development and maintanence of tissue and cancer is a disease of unregulated cell growth, disruptions in their signaling pathways likely play a role in the development of cancer. Therefore, mutations or interference of BMP signaling in stem cells may lead to the development of cancer, and studying BMP signaling in cancer syndromes may provide important insight into the development and progression of cancer and might help identify targets for the treatment of cancer.

BMPs and their receptors have been linked to the pathogenesis of some solid tumors. BMPRIA, BMPRII and BMP2 mRNA levels have been found upregulated in pancreatic cancers, 55% of which have biallelic loss of SMAD4 (Moskaluk et al., 1997; Zhang et al., 1997). BMP2 might promote pancreatic cancer progression by enhancing the growth of pancreatic cancer cells (Dicuonzo et al., 2001; Kleeff et al., 1999). BMP2 has also been shown to inhibit proliferation

and cause cell cycle arrest in the G1 phase of MKN74 gastric cells (Wen et al., 2004). Aberrant expression of BMP-2 occurs in approximately 98% of lung carcinomas, and BMP2 induces Id-1 expression in lung cancer cell lines through SMAD activation, but whether BMP2 is growth proliferative or suppressive is dependent on environmental conditions (presence or absence of serum) (Langenfeld et al., 2005).

Brubaker et al. found that BMP2 growth inhibitory effects can be seen in the androgen-sensitive prostate cancer cell line LNCAP, but not in the androgen-insensitive PC-3 and DU145 cell lines (Brubaker et al., 2004). They also found that BMP2 treatment activated SMAD1 phosphorylation, up-regulated $p21^{CIP1/WAF1}$ and changed retinoblastoma (Rb) expression in LNCaP and PC-3 cells. Additionally, BMP2 treatment stimulated a 2.7-fold increase in osteoprotegerin (OPG), a molecule, which inhibits osteoclastogenesis production in PC-3 cells (Brubaker et al., 2004; Horvath et al., 2004).

The mRNAs for the BMP receptors and BMP ligands are greater in cells with high metastatic potential than in cells with less metastatic potential (Arnold et al., 1999). However, BMP2 inhibits the proliferation of breast cancer cell lines that express both SMAD1 and SMAD4 (CAMA-1, MCF7, MDA-MB-231, T-47D, ZR-75-1) and upregulates the cyclin dependent kinase inhibitor $p21^{WAF1}$ in these cells (Pouliot & Labrie, 2002). Ghosh-Choudhury et al. showed that BMP-2 inhibits estradiol-induced cyclin D1-associated kinase and CDK2 activity with concomitant

reduction of Rb phosphorylation (Ghosh-Choudhury et al., 2000a). Eng et al. found that BMP2 exposure in MCF7 breast cancer cells resulted in increased PTEN levels. These data indicate that BMP2 exposure can regulate PTEN protein levels by decreasing PTEN's association with the degradative pathway, in particular with the association of proteins UbCH7 and UbC9, two ubiquitin degradation pathway proteins via SMAD1 (Waite & Eng, 2003a).

BMP7 has been shown to induce p21 expression in various epithelial cells through SMAD4, but only to weakly suppress epithelial cell proliferation. It is believed this is because of concomitant upregulation of Id2, a cell cycle promoting factor by BMP7 (Pardali K, 2005).

**Juvenile Polyposis**

Juvenile Polyposis is a clinical syndrome affecting 1 in 100,000 people (Burt et al., 1990) and both sporadic and familial cases with autosomal dominance inheritance are found (Fogt et al., 2004). Patients with Juvenile Polyposis Syndrome, Cowden Syndrome (CS) and Bannayan Riley Ruvalcaba Syndrome (BRRS) all develop intestinal juvenile polyps. Patients with the Juvenile Polyposis Syndrome often have 50–200 polyps ranging from a few millimeters to a few centimeters in size distributed throughout the colon that will recur. Microscopically, the polyps are mucin-filled cystic dilations of the epithelial tubules with normal mucosa (Chow & Macrae, 2005) Juvenile polyposis kindred

have a 12-fold increased lifetime risk for developing colorectal cancer(Huang et al., 2000). Four common *BMPRIA* germline mutations have been described in JP leading to premature stop codons and subsequent inactivation of *BMPRIA* (Howe, 2001). Somatic mutations in SMAD4 have been found to be present in approximately 20% of patients with juvenile polyposis (Friedl et al., 1999; Howe et al., 2004; Zhou et al., 2001b). Mutations in SMAD1, SMAD2, SMAD3, SMAD5 and SMAD7 are not found in Juvenile Polyposis patients (Bevan et al., 1999; Roth et al., 1999), while mutations in BMPRIA are present in approximately 20% of patients (Howe et al., 2001). Additionally, the majority of juvenile polyps with wild type APC contain nuclear β-catenin (Iwamoto et al., 2005). PTEN is mutated in BRRS and CS, two syndromes characterized by the presence of hamartomatous gastrointestinal polyps (Eng, 2001; Waite & Eng, 2003b).

**Colon Cancer**

Colorectal cancer is the second deadliest cancer in the United States. Multiple mutations in the cellular growth and progression pathways lead to the advancement of colon cancer. In more than 50% of colon cancers, K-Ras, a member of the Ras family, is found with constitutively activating mutations at codon 12 and 13 as an early event in the progression of benign adenomatous polyps to colorectal cancer (Bos et al.; Derynck & Zhang, 2003; Rebollo & Martinez-A,

1999). Additionally mutations in the PI3 Kinase signaling pathway are found in over 32% of colon cancers (Samuels et al., 2004).

Another common occurrence in the progression of colon cancer is inactivation of TGFβ superfamily signaling (Carethers, 2003; Grady et al., 1998). Inactivation of TGFβ signaling occurs in ~80% of colon cancers(Grady et al., 1998). Inactivation of activin signaling via mutations in *ACVR2*, another TGFβ superfamily receptor, occurs in the majority of colon tumors with microsatellite instability (MSI) (Hempen et al., 2003; Jung et al., 2004). Additionally, approximately 15% of colorectal tumors have mutations in SMAD4 (Thiagalingam et al., 1996).

Recently, Hardwick *et al.* found that BMP2 inhibits normal colonic epithelial cell growth by promoting apoptosis and differentiation, and inhibiting proliferation (Hardwick et al., 2004). They also found that BMP2, BMPRIA, BMPRIB, BMPRII, phosphorylated SMAD1, and SMAD4 are expressed predominantly in mature colonocytes at the epithelial surface in normal adult human and mouse colon tissue samples (Hardwick et al., 2004). Karoui et al. investigated the role of chromosome 10q loss in colon cancer metastasis as BMPRIA and PTEN both lie on this chromosome. They found that 10q allelic loss is associated with approximately 25% of MSI negative colorectal tumors, but not with metastasis (Karoui et al., 2004).

Some publications have identified oncogenic β-catenin as a requirement for BMP-4 ligand expression and secretion in colon carcinoma cells . Additionally, colon cancer cell lines with mutated *APC* genes increase β-catenin levels in the nucleus, which causes overexpression of BMP-4 (Kim et al., 2002).

Colon cancer is a result of uncontrolled cellular proliferation and dysregulation of cell death mechanisms (Carethers, 2003). While many of these pathways have been deciphered, the interactions of these pathways are likely a mechanism for tumor progression. Mutations in the TGF-β family have been described to play a key role in the pathogenesis of both microsatellite unstable (MSI) and chromosomal unstable (CIN) colorectal cancer (CRC). BMP is a TGF- β superfamily member that has been shown to be involved in prostate, pancreatic, and breast carcinomas, as well as the colorectal cancer predisposition syndrome Juvenile Polyposis. Patients with germline mutations in PTEN often exhibit these cancers. The BMP signaling pathway utilizes many of the same binding partners as other important pathways in the progression of colon cancer. Therefore, defining the role of BMP in the pathogenesis of colorectal cancer will provide insight and understanding into the progression of a disease that occurred in 150,000 and killed 56,600 people in the United States in 2005.

In this dissertation, we attempt to understand what role the BMP signaling pathway plays in colon cancer. We aimed to do this by: a) determining if the BMP pathway is intact in microsatellite stable and unstable colon cancer cell lines and

tissues, b) determining if the Ras pathway affects BMP signaling, and c) examining SMAD4-independent means of BMP signaling. In the rest of this dissertation, the results of these experiments will be presented and the implications of these results and future research will be discussed.

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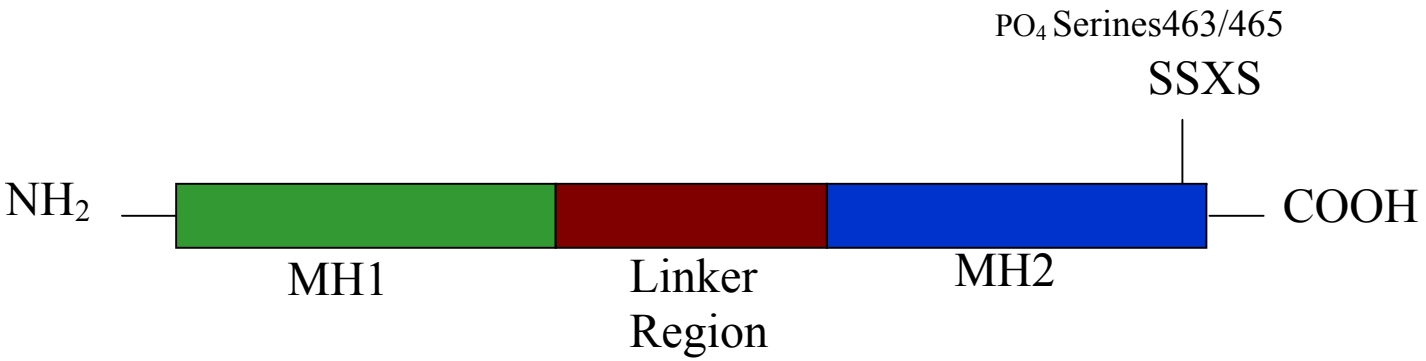


**Figure 1.1**. Schematic of Receptor SMAD Protein Phosphorylation Sites

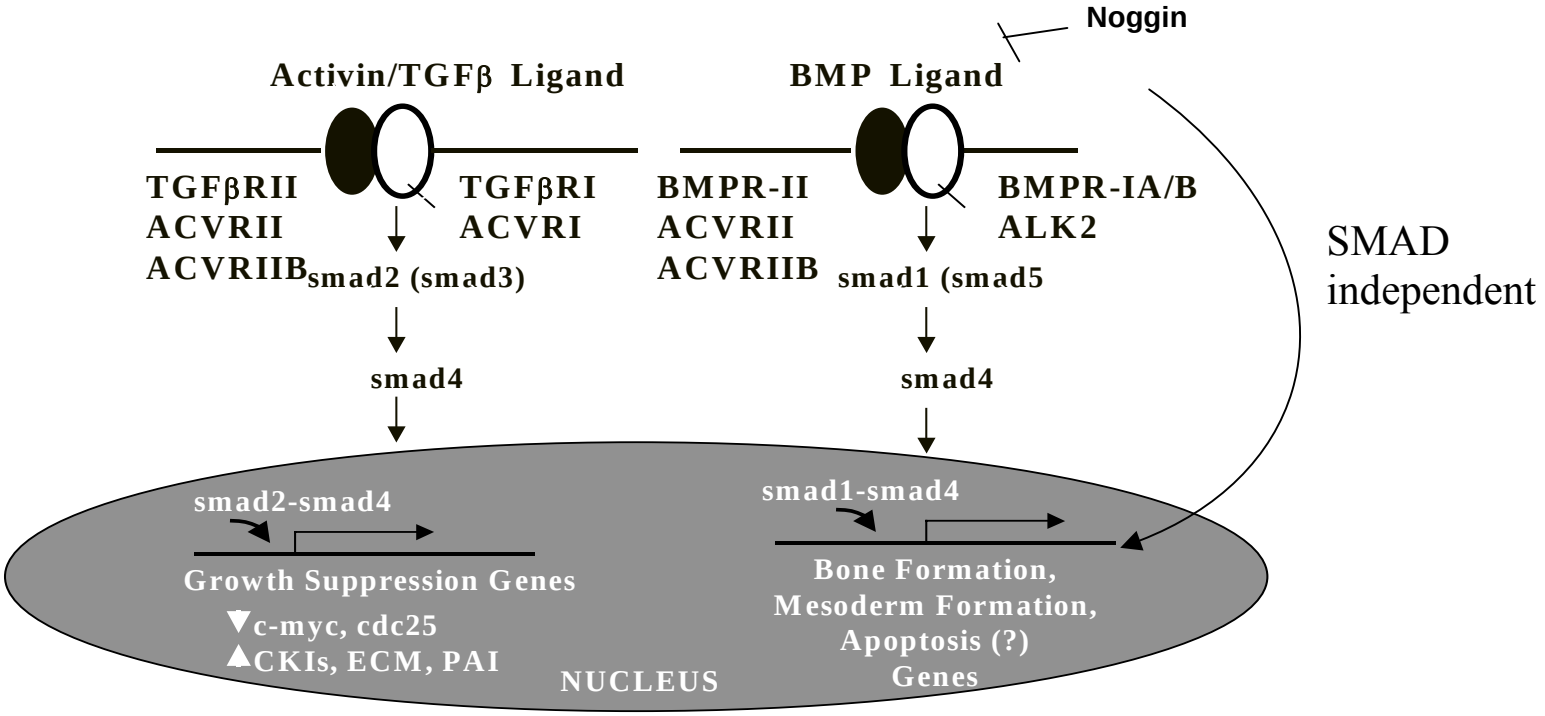


**Figure 1.2**. Transforming Growth Factor β Superfamily SMAD Signaling Pathways

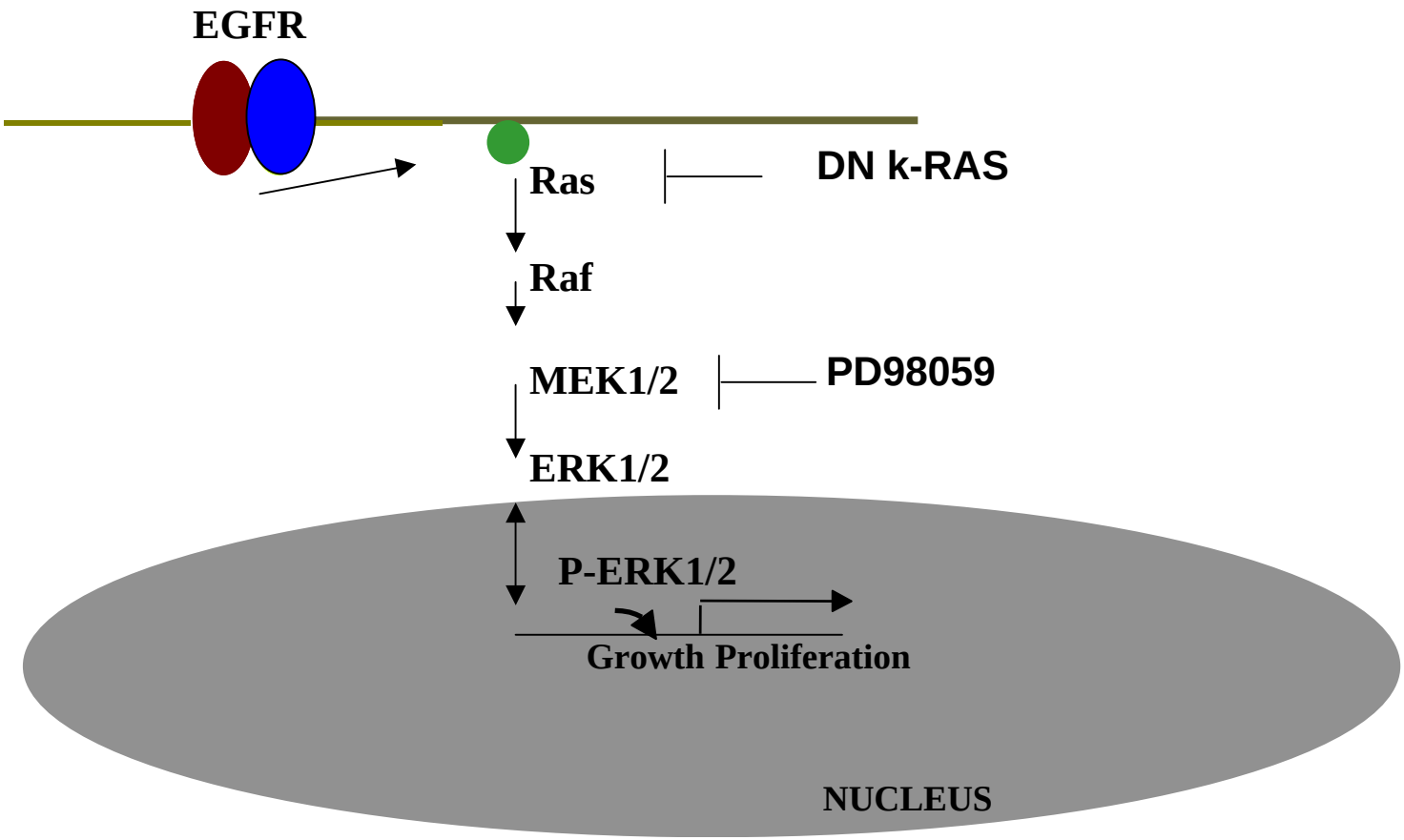


**Figure 1.3**. Ras Signaling Pathway

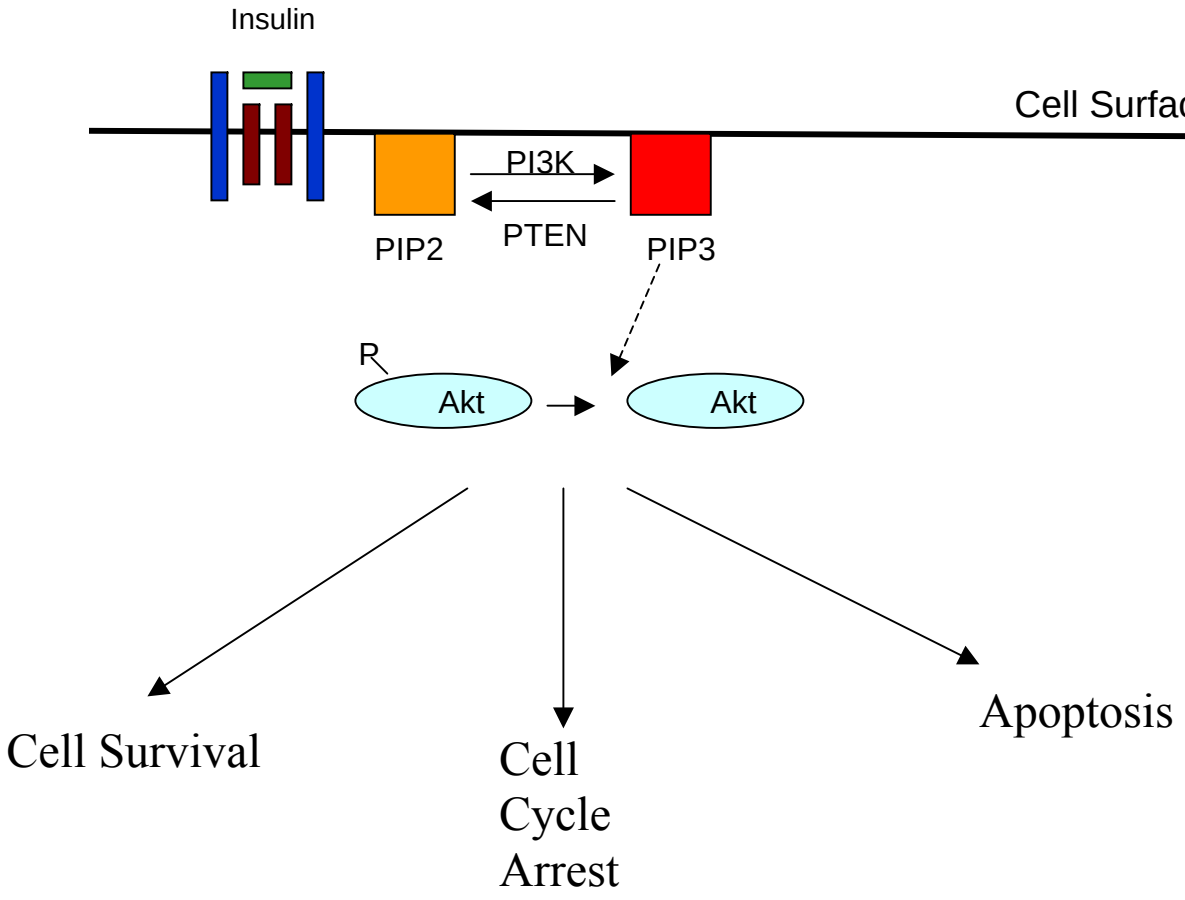


**Figure 1.4**. PI3 Kinase Signaling Pathway.

# Chapter 2:

# Materials and Methods

**Cell Culture**

HCT116, its derivatives, and SW480 cell lines were maintained in Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen Corporation, Carlsbad, CA) with 10% fetal bovine serum and penicillin G/streptomycin (Invitrogen Corporation, Carlsbad, CA. G418 was added to cultures of HCT116+chr2 and +chr3 to maintain the transferred chromosome. FET cells were maintained in Dulbecco's Modified Eagle Medium: Nutrient Mix F-12 (D-MEM/F-12) (1X), liquid, 1:1, with L-glutamine and HEPES buffer (Invitrogen Corporation, Carlsbad, CA) with 10% fetal bovine serum and penicillin G/streptomycin (Invitrogen Corporation, Carlsbad, CA).

**Nuclear/Cytoplasmic Fractionation, Immunoprecipitation, and Immunoblotting**

We separated the nuclear and cytoplasmic fractions with hypotonic lysis buffer (10mM TrisHCl pH 7.4, 3mM $MgCl_2$, 0.2% NP-40, 0.1mM EDTA, 40 mM NaF, 5mM glycerophosphate, 10μg/ml aprotinin, 5 μg/ml leupeptin, 1mM sodium orthovanadate, 0.5 mM PMSF) and RIPA buffer (1% NP40, 0.1% SDS, 1% DCA, 0.15 M NaCl, 50 mM TrisHCl pH 7.2, 1 MM PMSF). Immunoprecipitation of the receptors was performed by overnight incubation by rotary rotation with BMPRIA antibody with 500 μg of cell extract at 4°C. Protein Agarose A beads (Upstate, Lake Placid, NY) were added the mixture was further rotated for 3 hours at 4°C.

After washing with PBS, the antibody-protein complex was denatured at 100°C for 5 min. and the protein loaded onto an 8.5% polyacrylamide gel. After electrophoresis, the proteins were transferred onto a nylon membrane, blocked for 1 hr with 5% milk, and probed overnight with primary antibody at 4°C. Blotting was done with antibodies to BMPRIA 1:150, total SMAD1 1:300, SMAD4 1:400, histone 1:1000 (SC 5676, SC 7965, SC 7966, and SC 8030 respectively, Santa Cruz Biotechnology, Santa Cruz, CA), and phospho-SMAD1 1:400 (06-702, Upstate, Lake Placid, NY). Blotting was done with antibodies to total SMAD1 1:300, SMAD4 1:400, histone 1:1000 (SC 7965, SC 7966, and SC 8030 respectively, Santa Cruz Biotechnology, Santa Cruz, CA), phospho-SMAD1 1:400 (06-702, Upstate, Lake Placid, NY), p21 1:200 (OP64 Calbiochem, San Diego, CA), and PTEN 1:200 (SC7974, Santa Cruz Biotechnology, Santa Cruz, CA). The next day, several PBS-Tween 0.1% washes were performed along with appropriate secondary antibody incubation. Blotted proteins were detected with horseradish peroxidase-linked secondary antibodies (Sigma, St. Louis, MO) followed by ECL detection (Amersham, Little Chalfont, UK). The next day, several PBS-Tween 0.1% washes were performed along with appropriate secondary antibody incubation. Blotted proteins were detected with horseradish peroxidase-linked secondary antibodies (Sigma, St. Louis, MO) followed by ECL detection (Amersham, Little Chalfont, UK).

**Total Cell Lysis, and Immunoblotting**

Cells were lysed using total lysis buffer lysis buffer (12mM TrisHCl pH 8.3, 100mM NaCl, 1% SDS, 1% DCA, 1% Triton X-100, 2mM EDTA, 10μg/ml aprotinin, 10 μg/ml leupeptin, 50μM DTT, and 2mM PMSF). The protein was denatured at 100°C for 5 min. and the protein loaded onto an 15% polyacrylamide gel. After electrophoresis, the proteins were transferred onto a nylon membrane, blocked for 1 hr with 5% milk, and probed overnight with primary antibody at 4°C. Blotting was done with antibodies to p21 1:200 (OP64 Calbiochem, San Diego, CA), and PTEN 1:200 (SC7974, Santa Cruz Biotechnology, Santa Cruz, CA). The next day, several PBS-Tween 0.1% washes were performed along with appropriate secondary antibody incubation. Blotted proteins were detected with horseradish peroxidase-linked secondary antibodies (Sigma, St. Louis, MO) followed by ECL detection (Amersham, Little Chalfont, UK).

**Luciferase Assays**

Transient transfection of colon cancer cells with the BRE-Luc plasmid (a gift from Dr. Peter ten Dijke, Netherlands Cancer Institute, Amsterdam) was done to assess the effects of BMP on BMP-specific transactivation. The pWWP-luc plasmid (a gift from Burt Vogelstein, Johns Hopkins University, Baltimore, Maryland) was transfected to assess the effects of BMP on $p21^{WAF1}$ transactivation,

and the PTEN-luc plasmid was used to assess the effects of BMP2 on PTEN transactivation. Reporter vectors (0.75μg/ml) and the pRL-TK vector (20 ng/mL) are transiently delivered by Transfectin (Promega, Madison, WI) in 12-wells plates with a ratio of 3:1 of vector to transfection reagent in OPTI-MEM reduced serum free media (GIBCO Carlsbad, CA). Two hours post-transfection, 1ml of complete media was added per well and twelve to 16 hours post-transfection, cells were treated with 50 ng/ml of BMP2 or BMP7. Luciferase activity was measured by a dual-luciferase kit (Promega, Madison, WI) 20 to 24 hours after the treatment, and normalization was performed using the Renilla luciferase activity.

**Transfections**

DN k-RAS (a generous gift from Dr. Rik Derynck), SMAD4, dominant negative (DN) BMPR1A, or constitutively-active (CA) BMPR1A were transiently delivered by Transfectin (Promega, Madison, WI) at a ratio of 3:1 of vector to transfection reagent and transfecting 1 μg/mL of SMAD4 vector (a generous gift from Dr. Masayuki Funaba, Azabu University School of Veterinary Medicine in Japan) or 3 μg/mL of DN BMPR1A, DN kRAS or CA BMPR1A in OPTI-MEM reduced serum free media (GIBCO Carlsbad, CA). After 2-3 hours, IMDM with FBS and penicillin G/streptomycin was added to the transfected cells. Two hours post-transfection, complete media was added, and later used in the experiments. After 2-3 hours, IMDM with FBS and penicillin G/streptomycin was added to the

transfected cells. Two hours post-transfection, complete media was added, and later used in the experiments.

**MTT Assay**

The effect of BMP treatment on cell growth was assessed by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) based metabolic assay. Cells were seeded in 48-well plates at a density of 10 to 20,000 cells/well in 0.4 ml of culture medium supplemented with BMP 2 or BMP7 [100 ng/ml]. Metabolic activity, corresponding to growth, was assayed after 2 or 4 days of incubation at fixed intervals for MTT-dependent absorbency. For this, cells were stained 3 hrs with MTT dye, the reaction product released by lysis with SDS, and absorbency detected at 570λ using Beckman-Coulter DU640B spectrophotometer (Beckman-Coulter, Fullerton, CA).

**Cell Counting for Growth**

Cells were seeded at a density of 10,000 cells per well and 24 hrs. later were treated with 100 ng/ml BMP2 or BMP 7 in the presence of FBS. After 48 and 96 hours, cells were lysed in 0.5 ml of 0.05% trypsin and counted using a hemocytometer.

**Wound Closure Assay**

Cells were plated in six well plates and grown to 95% confluency. A wound was then created in the shape of a cross with a plastic pipet tip. The media was replaced with fresh media and the cells were photographed using a Carl Zeiss Axiovert 200 microscope (Carl Zeiss, Thornwood, NY). The cells were then treated with 100 ng/ml of BMP2, BMP7, or 300 ng/ml of Noggin. Every 18 hours thereafter, the media was refreshed and the cells were photographed and retreated. All pictures were taken from the same location on the plates at the top of the intersection of the cross.

**Invasion Assay**

The invasion assay was performed on SW480 cells using the QCM™ ECMatrix™ Cell Invasion Assay, Fluorimetric (cat. #: ECM555) (Chemicon, Temecula, CA). Briefly cells were plated in a 96 well plate insert with and extracellular matrix bottom in 1% FBS media. The insert was then put into another 96-well plate with 10% FBS. Cells were treated with various 50 ng/ml of BMP2 and allowed to invade through the matrix for 24 hours before being analyzed using a fluorescent plate reader.

**Total RNA Extraction and Semi-Quantitative Reverse Transcriptase-Polymerase Chain Reaction**

Total RNA extraction was performed using Trizol reagent (Invitrogen Corporation, Carlsbad, CA). Cells grown on 6-well plates were lysed with trizol (1 mL/well) and were combined with chloroform and mixed. Supernatants were then precipitated with isopropanol, and the RNA pellets were washed with 75% ethanol and air-dried, then was resuspended in water. Two micrograms of total RNA was converted into cDNA by reverse transcriptase and amplification of BMP2 and BMP7 (SuperScript II, Invitrogen Corporation). Briefly, following inactivation at 65°C for 10 min, 1 μL of the reaction mixture was incubated in buffer containing 0.2 mM concentrations of dATP, dCTP, dGTP, dTTP, 0.2 μM concentrations each of oligonucleotide primers, 3 mM $MgCl_2$ and a 10X buffer consisting of 200 mM Tris-HCl (pH 8.0), 500 mM KCl, and 1 Taq polymerase. The following primers were designed to amplify BMP2 and BMP7: BMP2, forward 5'-CCCAGCGTGAAAAGAGAGAC-3' and reverse 5'GAGACCGCAGTCCGTCTAAG-3'; BMP7, forward 5'-TCGTGGAACATGACAAGGAA-3' and reverse 5'-CTGATCCGGAACGTCTCATT-3'. Primers for p21/Waf1 were as follows: forward 5'CAGGGGACAGCAGAGGAAGA-3' and reverse 5'-TTAGGGCTTCCTCTTGGAGAA-3'. Primers for PTEN are forward 5'-GGACGAACTGGTGTAATGATATG-3' and reverse 5'-

TCTACTGTTTTTGTGAAGTACAGC-3'. *GAPDH* served as a loading control forward 5'-ACCACAGTCCATGCCATCAC-3' and reverse 5'-TCCACCACCCTGTTGCTGTA-3'. PCR was performed as follows: denaturation at 95°C for 3 min and 35 cycles of 94°C for 30 s, 55°C for 30 s, and 74°C for 4 min for BMP7 and GAPDH, BMP2: denaturation at 95°C for 3 min and 40 cycles of 94°C for 30 s, 57°C for 30 s, and 74°C for 4 min, p21: denaturation at 95°C for 3 min and 40 cycles of 94°C for 30 s, 55°C for 30 s, and 74°C for 4 min., and PTEN: denaturation at 95°C for 3 min. and p21: denaturation at 95°C for 3 min and 40 cycles of 94°C for 30 s, 55°C for 30 s, and 74°C for 4 min.

**Cell Cycle Analysis**

Cells were grown on 10 cm dishes until 50% confluent. After 24 hours of serum starvation, 10% serum was added containing 50 ng/ml of BMP2 or BMP7 for 48 hrs. The cells were harvested using 0.05% trypsin, washed with PBS and resuspended in 0.6 ml of PBS and 1.0 ml of 100% ethanol. The cells were fixed overnight at 4°C. The next day, the cells were centrifuged, washed in PBS, and centrifuged again for 5 min. The pellet was resuspended in 0.5 ml of a PBS solution containing 40 units/ml RNase A and 50 µg/ml propidium iodide, incubated at 37°C for 1 hr, then placed on ice until analysis with a Beckman-Coulter Elite Flow Cytometer Multicycle (Phoenix Flow, San Diego, CA).

**Propidium Iodide Viability**

For viability assays, cells were serum starved 30 min., and then treated with 50 ng/ml of ligand for 48 hrs. Cells were then harvested with 0.05 % trypsin, centrifuged at 1500 rpm for 5 min. and resuspended in 0.3 ml PBS. Ten μg/ml of propidium iodide was added to the cells, and the cells were placed on ice until analysis with a Beckman-Coulter Elite Flow Cytometer (Phoenix Flow, San Diego, CA).

**Immunohistochemical Analysis**

Under IRB approval (UCSD protocol #050958XT), ten random microsatellite stable and three random microsatellite unstable slides from a population-based study (35) containing colon cancer tissue were deparaffinized in xylene and rehydrated in graded alcohols to water. The slides were immersed in sodium citrate buffer (pH 6.0), and heated in a microwave for 4 minutes for 4 times for antigen retrieval. Slides were then processed using a DAKO® Signal Catalyzed Amplification (CSA) System (DAKO Corporation, Carpinteria, CA). Endogenous peroxidase activity was blocked by incubation with 3% $H_2O_2$. Ten percent goat serum was added for 15 minutes to block nonspecific protein binding. Slides were incubated overnight with primary antibody (BMPRIA 1:150 (SC 5676, Santa Cruz Biotechnology Santa Cruz, CA), BMP2 4.5 μg/ml, BMP7 25 μg/ml (AF 355 and AF354, R&D Systems Minneapolis MN), pSMAD1 1:100 (AB 3848, Chemicon

Temecula CA)), and then rinsed with phosphate buffered saline-tween 0.1% (PBS-T). Biotinylated secondary antibody was added for 15 minutes followed by incubation with peroxidase-labeled streptavidin for 15 minutes at room temperature. The sections were washed with PBS-T, incubated with DAB and $H_2O_2$ for 1 minute, lightly counterstained with hematoxylin, dehydrated in graded alcohols, cleared in xylene, and coverslipped. A single gastrointestinal pathologist reviewed all sections and immunostains.

**Deconvolution Digital Immunofluorescent Microscopy**

Cells were plated in four chamber slides (Nunc Inc. Naperville, IL) at 1000 cells/ chamber. Once cells became 80% confluent on the coverslip, at approximately 48 hrs after plating, the cells were exposed to chemical inhibitor or media alone for 30 min followed by the addition of 100 ng/ml BMP2. After one hour of exposure, the slides were placed at 4ºC and washed 3 times with phosphate buffered saline and then fixed in 3.7 % formaldehyde in the same buffer at room temperature for 30 minutes and permeabilized with 0.3% Triton X-100. The slides were then blocked with 5 % bovine serum albumin in phosphate buffered saline for 1 hour. The anti-phospho-SMAD1 primary antibody (Chemicon, Temecula, CA) was diluted 1:100 in 5 % bovine serum albumin in phosphate buffered saline and added for two hours at room temperature. Biotin SP-conjugated Affinity Pure Donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) was then

added at a 1:250 dilution for 2 hours in 5 % bovine serum albumin in phosphate buffered saline and incubated at room temperature. Hoescht 33342 dye (Molecular Probes, Eugene, OR) was also added to the secondary antibody solution at a concentration of 1:10000. Slides were then stained with Streptavadin Alexa 488 (Molecular Probes, Eugene, OR) at 1:1000 in 5 % bovine serum albumin in phosphate buffered saline and incubated at room temperature for 2 hours. FET cells were probed with phospho-SMAD1 Hoechst stain (blue) was used to stain the nucleus and phospho-SMAD1 (green) was illuminated using Alexa488 fluorescent dye.

Coverslips were then mounted on the glass slides using Gelvatol (Air Products and Chemicals, Inc., Allentown, PA). Images were captured with a DeltaVision Restoration microscope system (Applied Precision Inc., Issaquah, WA) using a Photometrics Sony Coolsnap HQ charged-coupled device (CCD) camera system attached to an inverted, wide-field fluorescent microscope (Nikon TE-200). Optical sections were acquired using a 60x Nikon (NA 1.4) oil immersion objective in 0.2 μm steps in the z-axis. Images were saved, processed, and analyzed on Silicon Graphics Workstations (O2, Octane) using the DeltaVision software package *Softworx* (Version 2.50). The images in each panel of the figures in this paper are representative of 3 images taken from each of 3 independent experiments. Images are normalized to the autofluorescence of unstained cells and cells stained with only the secondary antibody and Streptavadin Alexa 488 probe.

Two independent observers counted the number of fluorescent dots in the nucleus and the cytoplasm to quantify the data.

**Statistical Analysis**

Statistical significance was determined using either the student's t-test or two-factor without replication ANOVA. Probability values less than 0.05 were considered to be significant.

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# Chapter 3:

# Bone Morphogenetic Protein Signaling and Growth Suppression in Colon Cancer

## Introduction

Bone Morphogenetic Protein (BMP) is a member of the TGFβ superfamily known to regulate cell proliferation, apoptosis, and differentiation, and participates in the mesenchymal development of most tissues and organs in vertebrates. However, its role in epithelial growth regulation is not well understood.

Recently, Hardwick *et al.* found that BMP2 inhibits normal colonic epithelial cell growth by promoting apoptosis and differentiation, and inhibiting proliferation (Hardwick, 2004). They also found that BMP2, BMPRIA, BMPRIB, BMPRII, phosphorylated SMAD1, and SMAD4 are expressed predominantly in mature colonocytes at the epithelial surface in normal adult human and mouse colon tissue samples (Hardwick, 2004).

Juvenile Polyposis Syndrome (JP) is an autosomal dominant gastrointestinal hamartomatous polyposis syndrome that increases the afflicted patient's risk for developing colon cancer ~12-fold. Germline mutations in the tumor suppressor *SMAD4* and *BMPRIA* have been described in JP patients (Howe, 1998; Zhou, 2001). A small percentage of JP kindreds have also shown germline mutations in *PTEN* (Huang, 2000). Additionally, Haramis *et al.* found that inhibition of BMP signaling by conditional knockout of *BMPRIA* results in the formation of numerous ectopic crypts, which mimic the intestinal histopathology of JP (Haramis, 2004).

Colon cancer develops as a result of uncontrolled cellular proliferation and dysregulation of cell death mechanisms, and inactivation of TGFβ superfamily

signaling appears to play key roles (Carethers, 2003). Inactivation of TGFβ signaling occurs in ~80% of colon cancers (Grady, 1998). Inactivation of activin signaling via mutations in *ACVR2*, another TGFβ superfamily receptor, occurs in the majority of colon tumors with microsatellite instability (Jung, 2004; Hempen, 2003). As patients with the colon cancer predisposition syndrome JP develop germline mutations in key BMP signaling molecules, and the effects of BMP signaling in colon cancer are largely unknown, we aimed to answer two questions: 1) is BMP signaling disrupted in colon cancer like its TFGβ and activin counterparts, and 2) if not, does BMP signaling confer growth control in colon cancer cells? We found that BMP signaling is intact in human colon cancer specimens and in several cell lines, and is moderately growth suppressive.

**Results**

The BMPRIA receptor is present and the SMAD signaling is intact in the majority of colon cancer cells

To investigate whether the BMP pathway is perturbed in colon cancer cell lines, we first sought to examine whether the BMPRIA receptor is present, as this receptor is often mutated in colon cancer prone JP patients. All of the colon cancer cell lines examined expressed BMPRIA at the protein level (**Figure 3.1A**). We next examined whether the receptor was functional. We treated cells with BMP2 or

BMP7, and examined SMAD1 phosphorylation by using a phospho-specific SMAD1 antibody after nuclear and cytoplasmic fractionation. In response to BMP ligand, the HCT116, HCT116+chr2, and HCT116+chr3 cancer cell lines phosphorylated and translocated pSMAD1 to the nucleus. Total SMAD1 and SMAD4 were also present in both the cytoplasm and nucleus (**Figure 3.1 B-D**).

BMP-induced specific transcription is intact in colon cancer cells

Transcriptional activation due to BMP2 or BMP7 stimulation was determined with the use of the BMP-specific SMAD-induced luciferase reporter, BRE-Luc (a generous gift from Dr. Peter ten Dijke) (Korchynskyi, 2002). Transcriptional activation by BMP increased over untreated controls, but varied among the cell lines: HCT116 and HCT116+chr3 cells transcriptional activity increased 3-5 fold, whereas HCT116+chr2 cell lines increased 8-13 fold (**Figure 3.2 A-B**).

BMP Ligands are Moderately Growth Suppressive in Colon Cancer Cells

Cell growth was indirectly evaluated by MTT assay and directly by cell counting. HCT116+chr2 and HCT116+chr3 cells demonstrated modest but significant decreases in growth when treated with 100 ng/ml of BMP2 or BMP7 for 48 hours as assessed by MTT assay (**Figure 3.3**). HCT116 cells also demonstrated significant decreases in growth when treated with BMP7, but not BMP2. Direct

cell counting of the cell lines after BMP treatment showed decreased growth of BMP-treated cells compared to untreated cells. We observed inhibition after 2 days of growth in the HCT116 +chr3 cells with either BMP2 or BMP7 ligand, but not the HCT116 or HCT116 +chr2 cells. HCT116 cells after BMP2 and HCT116+chr2 cells after BMP2 and BMP7 showed decreased cell growth after 4 days of ligand treatment **(Figure 3.4 A-C)**. Only HCT116 cells treated with BMP7 had some modest growth enhancement, which was reduced by day 4. Overall, our data are consistent with the MTT results.

Dominant negative BMPR1A reverses transcriptional activity and growth inhibitory effects of BMP ligands

We transfected the HCT116+chr2 and HCT116+chr3 cells with a *BMPRIA* dominant negative construct (DN-BMPR1A) and compared the results with a transfected BMPRIA constitutively active (CA BMPR1A) vector or mock vector (15). Post transfection, we treated the cells with BMP2 or BMP7, and assayed transcription via the BRE-luciferase assay. Transcriptionally, DN BMPR1A reduced the effect of BMP ligands compared to mock and CA BMPR1A transfections in HCT116+chr2 and HCT116+chr3 cell lines (**Figure 3.5 A-B**). Thus, DN BMPR1A transfection reduces BMP-SMAD mediated transcription in our cell models.

DN BMPR1A reversed the BMP-induced growth suppressive effects in our cell models. In both HCT116+chr2 and HCT116+chr3 cells, CA BMPR1A was more effective in reducing growth than exogenous BMP2 or BMP7 ligand treatment. However, the presence of DN BMPR1A attenuated or reversed BMP-induced growth suppression in both of these cell lines (**Figure 3.6 A-B**). Thus, we show reversal of BMP-induced growth suppression when signaling through BMPR1A is impaired, indicating that this receptor is a gateway for growth suppression.

BMP2 and BMP7 and alteration of the cell cycle

We examined the functional effects of BMP treatment on the cell cycle in the colon cancer cell lines. Despite intact BMP signaling and growth suppression, none of the HCT116 cell lines examined demonstrated significant alteration of the cell cycle (data not shown). Because p21 has been shown to be upregulated by BMP's in some model systems, we further examined whether $p21^{WAF1}$ was transcriptionally activated by BMP in cell lines by transfecting pWWP-Luc, a $p21^{WAF1}$-specific reporter plasmid. We found no upregulation in transcriptional activity of $p21^{WAF1}$ upon stimulation with BMP2 or BMP7 over controls in the HCT116 and HCT 116+chr2, or HCT116+chr3. (**Figure 3.7A)**. We also examined endogenous p21 RNA expression by Reverse Transcriptase PCR after BMP2 and

BMP7 treatment and found no change in p21 mRNA levels in any of the cell lines examined **(Figure 3.7 B-D)**.

Human Colon Cancer Specimens exhibit intact BMP signaling components

To assess the status of BMP signaling in primary human colon cancer specimens, we utilized immunohistochemistry to examine the presence or absence of the signaling molecules. We examined thirteen primary colon cancer tissues: three microsatellite instability-high and ten microsatellite stable. All of the cancers (and paired normal colonic tissue) expressed pSMAD1, BMPRIA, BMP2, and BMP7. Representative pictures of the immunohistochemical stains are shown (**Figure 3.8**).

**Discussion**

BMPs are known to play a role in tissue development, but until recently, little work has been done examining the significance of BMP signaling in cancer. Work by several groups has previously shown BMP signaling to affect epithelial cell growth (Brubaker, 2004; Hardwick, 2004; Pouliot, 2002). TGFβ and activin ligands from the same superfamily as BMP are known growth suppressors, and have shown to be inactivated in subsets of colon cancers (Jung, 2004; Grady, 1999; Markowitz, 1996; Hempen, 2003). BMP signaling can be inactivated by germline mutation of *BMPRIA* in the colon cancer predisposition syndrome, juvenile

polyposis (Zhou, 2001; Howe, 2001). Taken together, these facts led us to hypothesize that the BMP pathway might be affected in colon cancer cell lines and human colon cancer specimens without juvenile polyposis.

In juvenile polyps from patients with JP, loss of BMP signaling might contribute to the high risk of colon cancer observed over the lifetime of these patients. Given that BMP signaling appears to be growth suppressive, there are a few possibilities why BMP signaling remains intact in colon cancers. First, the degree of tumor suppression may be moderate and thus overcome by other genetic events that occur in colon cancers. Secondly, other signaling pathways may adversely affect BMP growth suppressive signaling, such that traditional SMAD signaling is abrogated without mutation of the components. These findings indicate further exploration is necessary of the pathways, and potential cross-talk between the pathways should be investigated.

**Acknowledgements**

The contents of Chapter 3, in part or full, are in press (**Beck SE**, Jung BH, Fiorino A, Gomez J, Del Rosario E, Cabrera BL, Huang SC, Chow JYC, and Carethers JM. "Bone morphogenetic protein signaling and growth suppression in colon cancer." Am J Physiol Gastrointest Liver Physiol. 2006 Jul;291(1):G135-45). Stayce Beck was the primary author of this chapter. John Carethers directed and supervised the writing of this chapter.

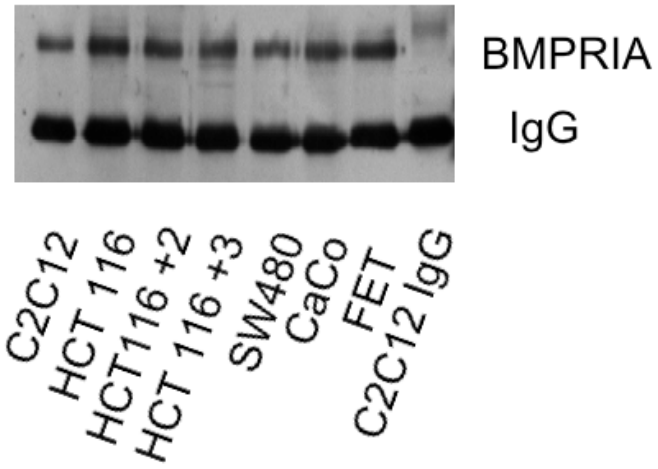



**Figure 3.1(*A*)**. Immunoprecipitation of BMPRIA in various colon cancer cell lines. The C2C12 mouse cell line is used as a positive control. FET and CaCo are additional colon cancer cell lines.

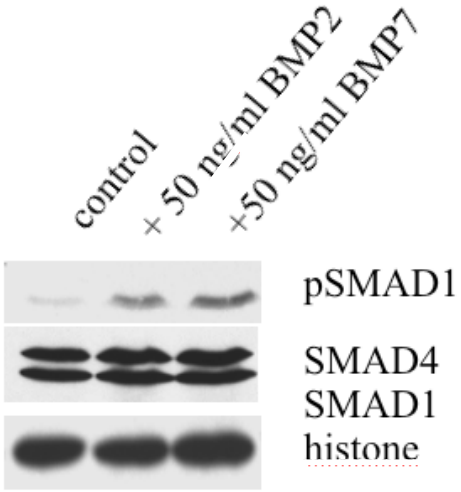



**Figure 3.1(*B*)**. Nuclear extracts of HCT116 when treated with BMP2 or BMP7. Membranes were blotted for pSMAD1, SMAD1, and SMAD4. Histone was used as a loading control for the nuclear fractionations.

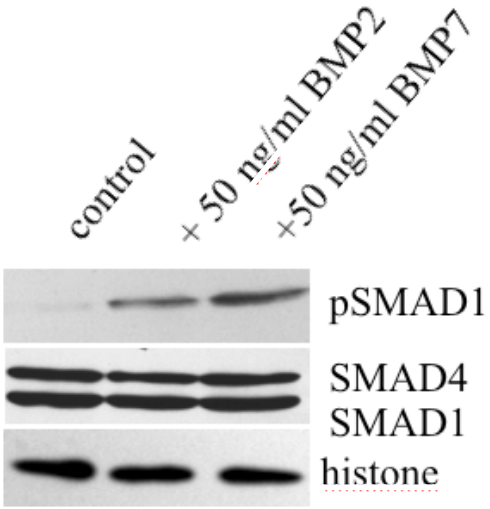



**Figure 3.1(*C*)**. Nuclear extracts of HCT116+chr2 cells when treated with BMP2 or BMP7. Membranes were blotted for pSMAD1, SMAD1, and SMAD4. Histone was used as a loading control for the nuclear fractionations.

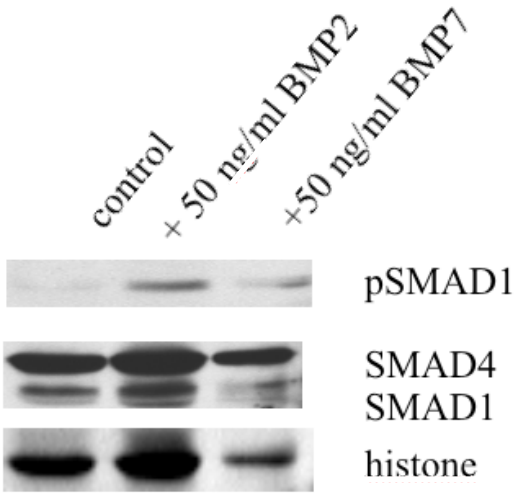



**Figure 3.1(*D*)**. Nuclear extracts of HCT116+chr3 cells when treated with BMP2 or BMP7. Membranes were blotted for pSMAD1, SMAD1, and SMAD4. Histone was used as a loading control for the nuclear fractionations.

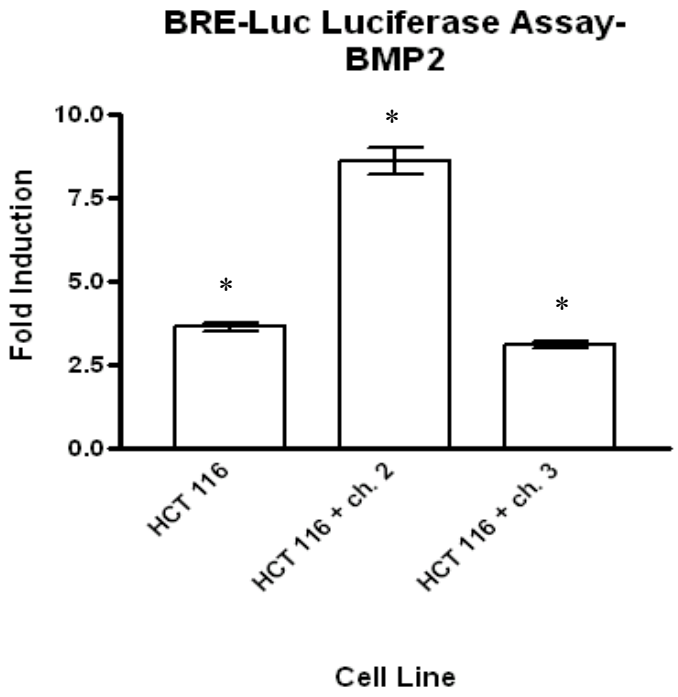



**Figure 3.2(*A*).** BMP-induced transcriptional activity in colon cancer cells. Fold induction of BMP2 treatment over no treatment (control) of BMP-induced SMAD transcriptional activity in HCT116 cell line derivatives.

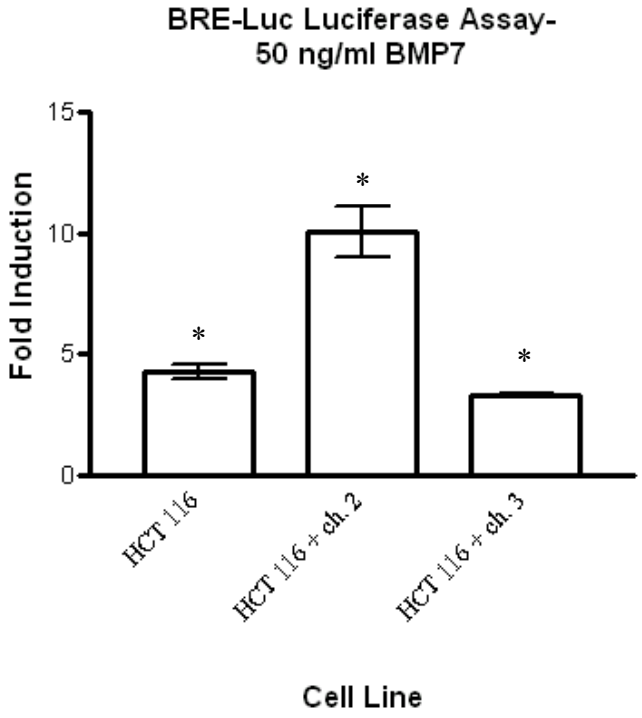



**Figure 3.2(*B*).** BMP-induced transcriptional activity in colon cancer cells. Fold induction of BMP7 treatment over no treatment (control) of BMP-induced SMAD transcriptional activity in HCT116 cell line derivatives.

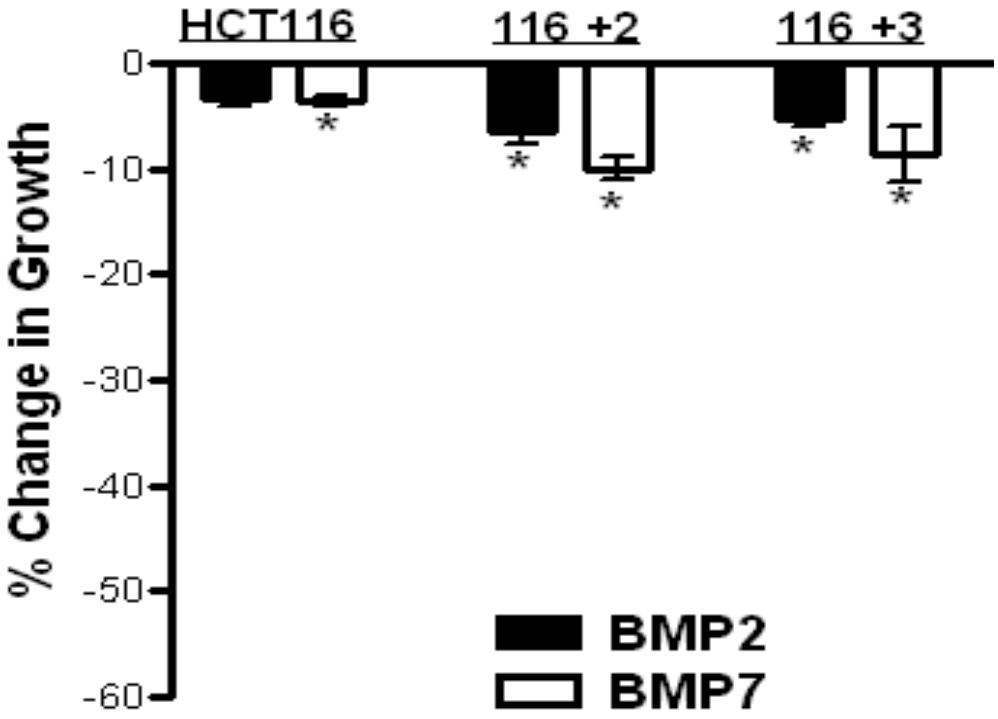



**Figure 3.3.** Effect of 48 hour BMP2 or BMP7 treatment on cell growth as assessed by MTT assay in HCT116, HCT116+chr2, and HCT116+chr3 cells. Two-factor with replications ANOVA was used to determine p-values (*$p<0.05$).

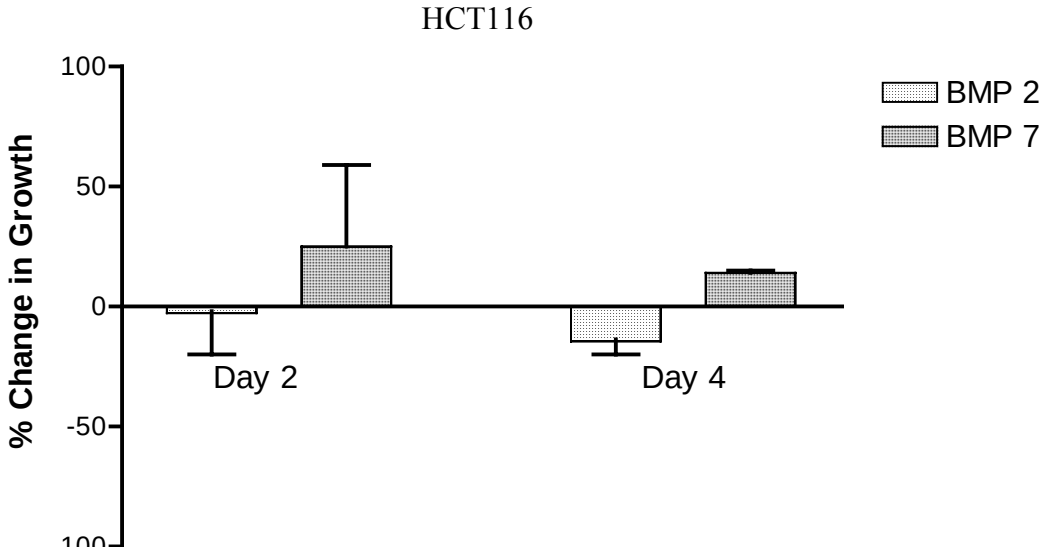



**Figure 3.4(*A*).** Effect of BMP2 or BMP7 treatment on cell growth as assessed by cell counting in HCT116 cells after 2 days and 4 days of BMP2 and BMP7 treatment.

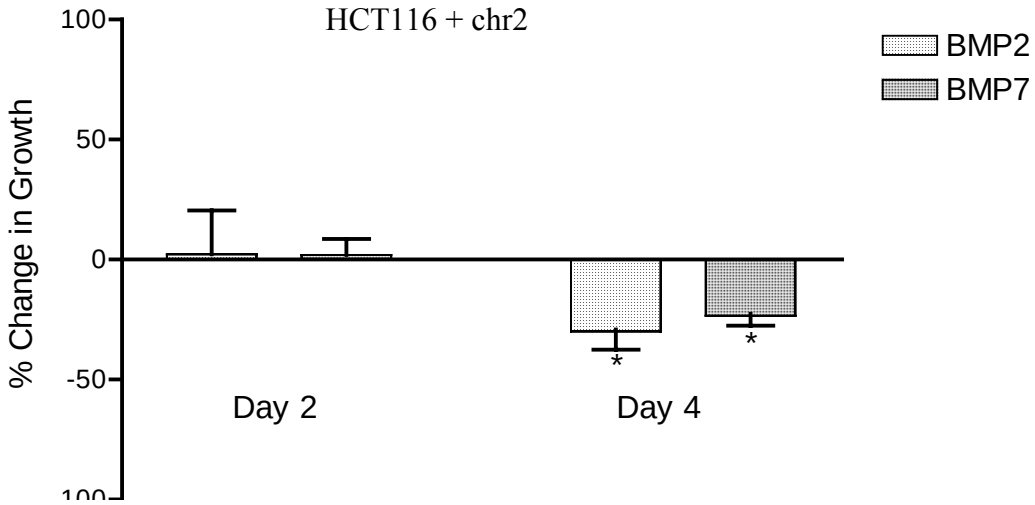



**Figure 3.4(*B*).** Effect of BMP2 or BMP7 treatment on cell growth as assessed by cell counting in HCT116+chr2 cells after 2 days and 4 days of BMP2 and BMP7 treatment.

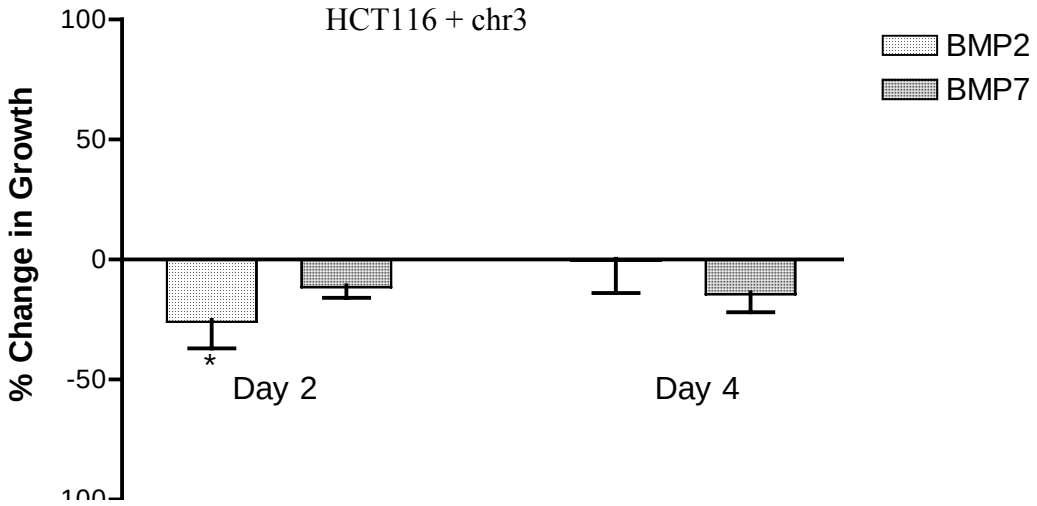



**Figure 3.4(*C*).** Effect of BMP2 or BMP7 treatment on cell growth as assessed by cell counting in HCT116+chr3 cells after 2 days and 4 days of BMP2 and BMP7 treatment.

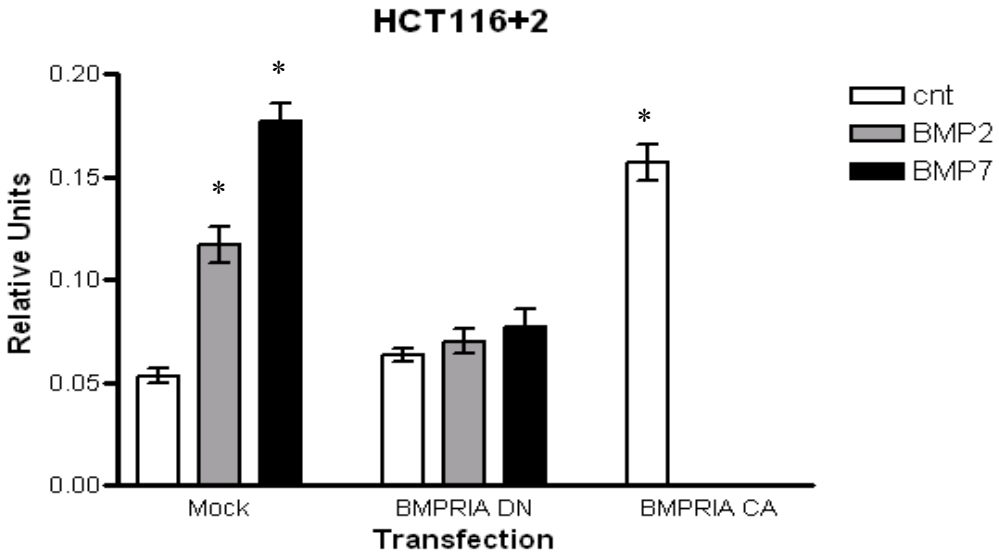



**Figure 3.5(*A*).** Relative amount of SMAD-induced transcriptional activity after BMP ligand treatment, and the effect of dominant negative (DN) BMPR1A transfection in HCT116+chr2 cells. Constitutively active (CA) BMPR1A is shown as a positive control in the absence of ligand treatment.

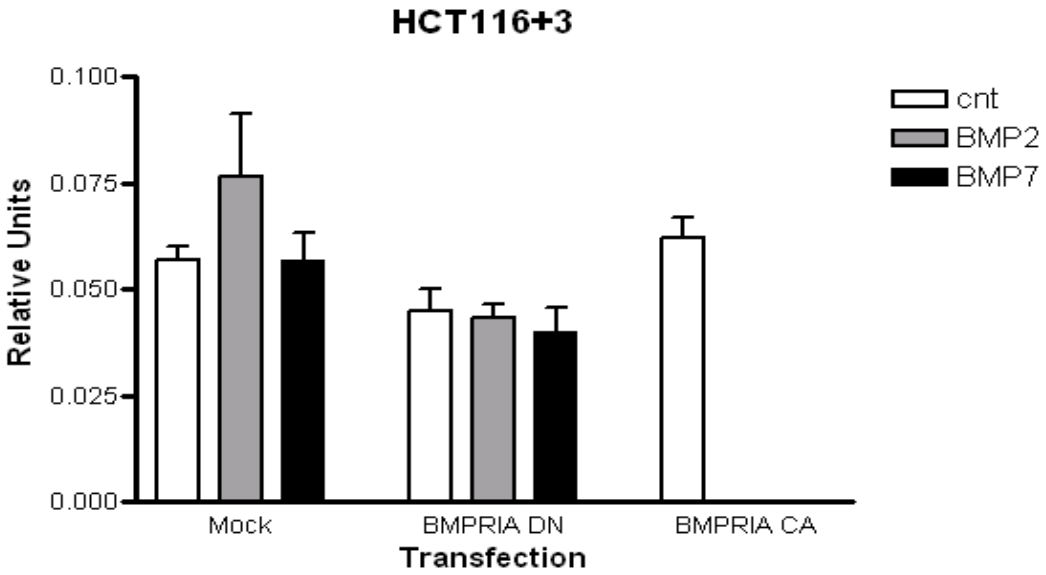



**Figure 3.5(*B*).** Relative amount of SMAD-induced transcriptional activity after BMP ligand treatment, and the effect of dominant negative (DN) BMPR1A transfection in HCT116+chr3 cells. Constitutively active (CA) BMPR1A is shown as a positive control in the absence of ligand treatment.

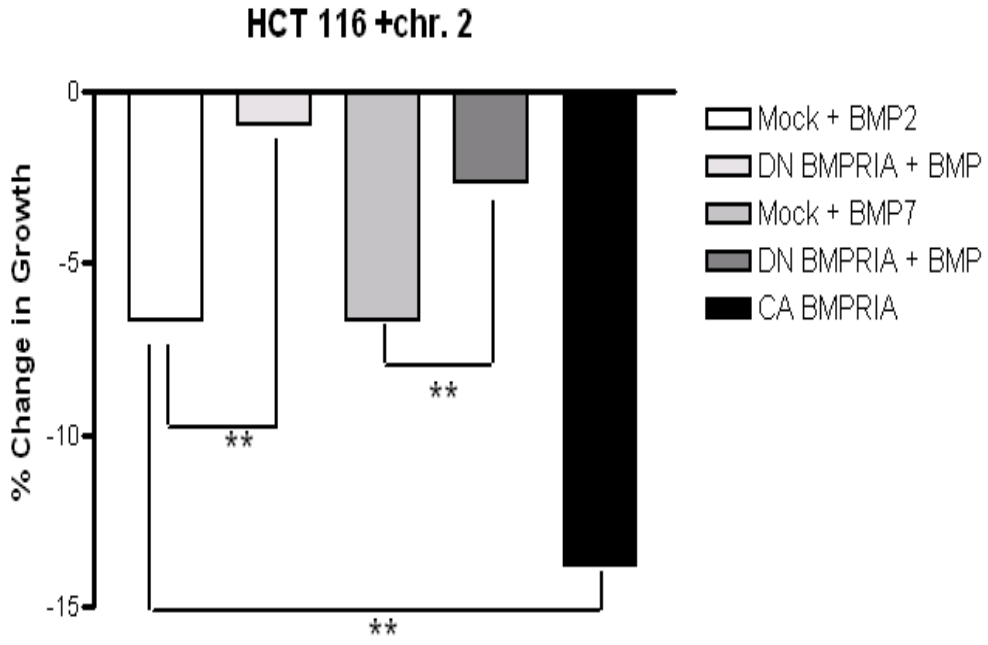



**Figure 3.6(*A*).** Dominant negative (DN) BMPR1A transfection reverses BMP-induced growth suppression as assessed by MTT assay in HCT116+chr2 cells. Two-factor with replications ANOVA was used to determine p-values (*$p<0.05$, **$p<0.01$).

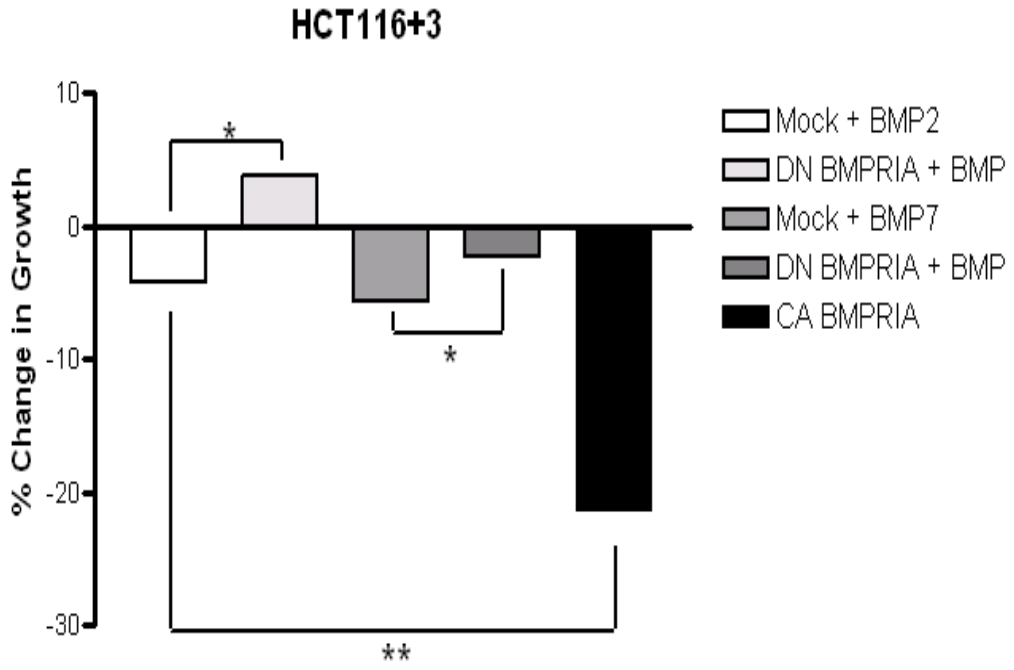



**Figure 3.6(*B*).** Dominant negative (DN) BMPR1A transfection reverses BMP-induced growth suppression as assessed by MTT assay in HCT116+chr3 cells. Two-factor with replications ANOVA was used to determine p-values (*$p<0.05$, **$p<0.01$).

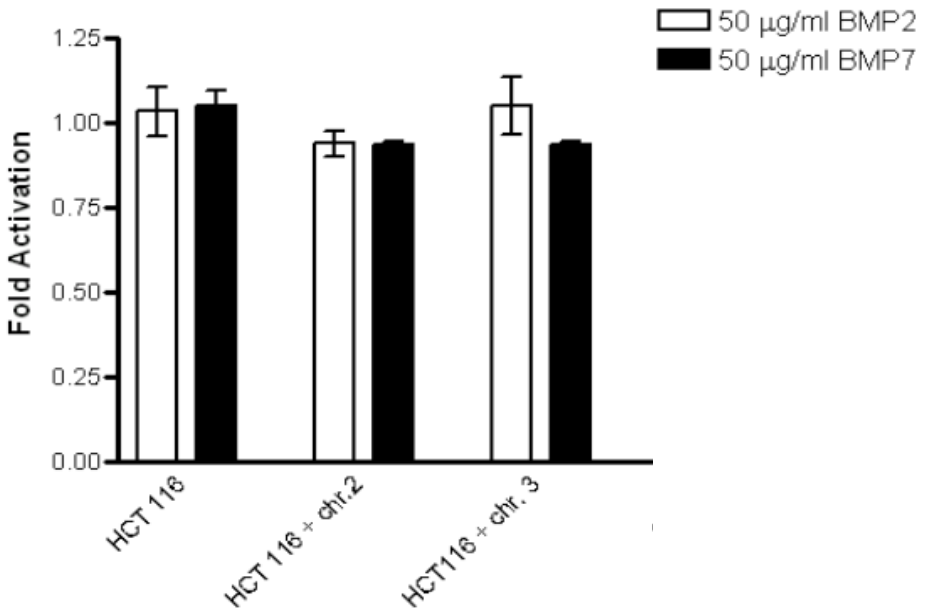



**Figure 3.7(*A*).** Results of p21 luciferase assay using pWWP-luc plasmid on HCT116, HCT116+chr2, and HCT116+chr3.

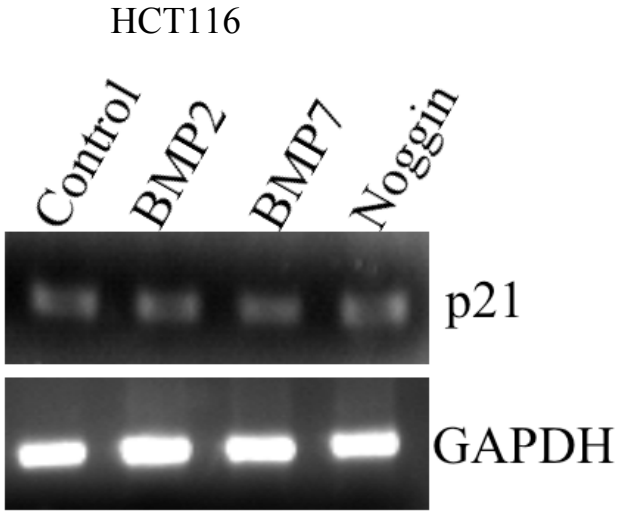



**Figure 3.7(*B*).** Effect of BMPs on p21 mRNA expression in HCT116 after treatment with BMP2, BMP7 and Noggin.

HCT116 + chr2

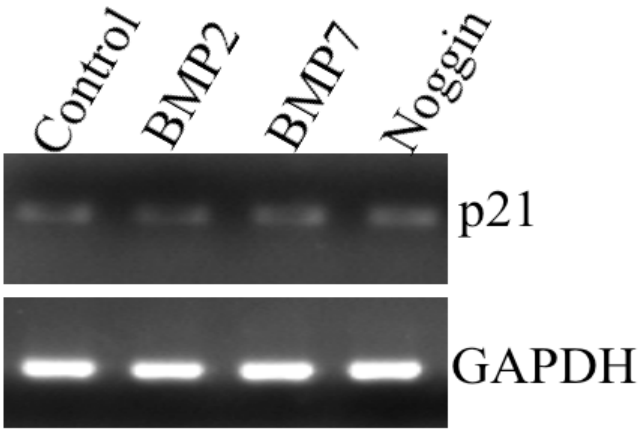



**Figure 3.7(*C*).** Effect of BMPs on p21 mRNA expression in HCT116+chr2 after treatment with BMP2, BMP7 and Noggin.

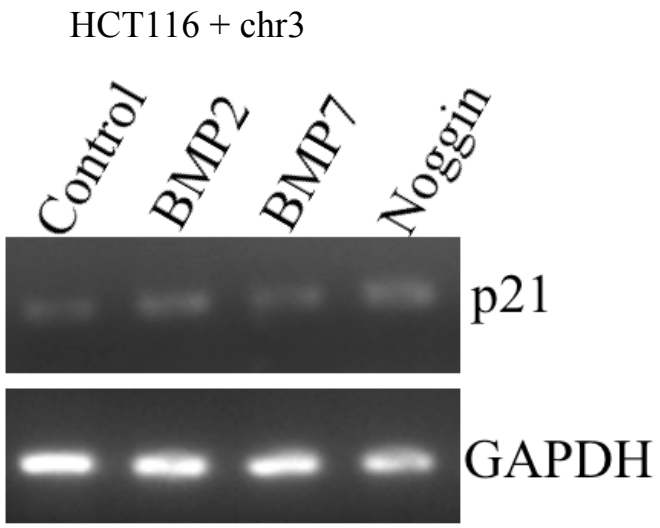



**Figure 3.7(*D*).** Effect of BMPs on p21 mRNA expression HCT116+chr3 after treatment with BMP2, BMP7 and Noggin.

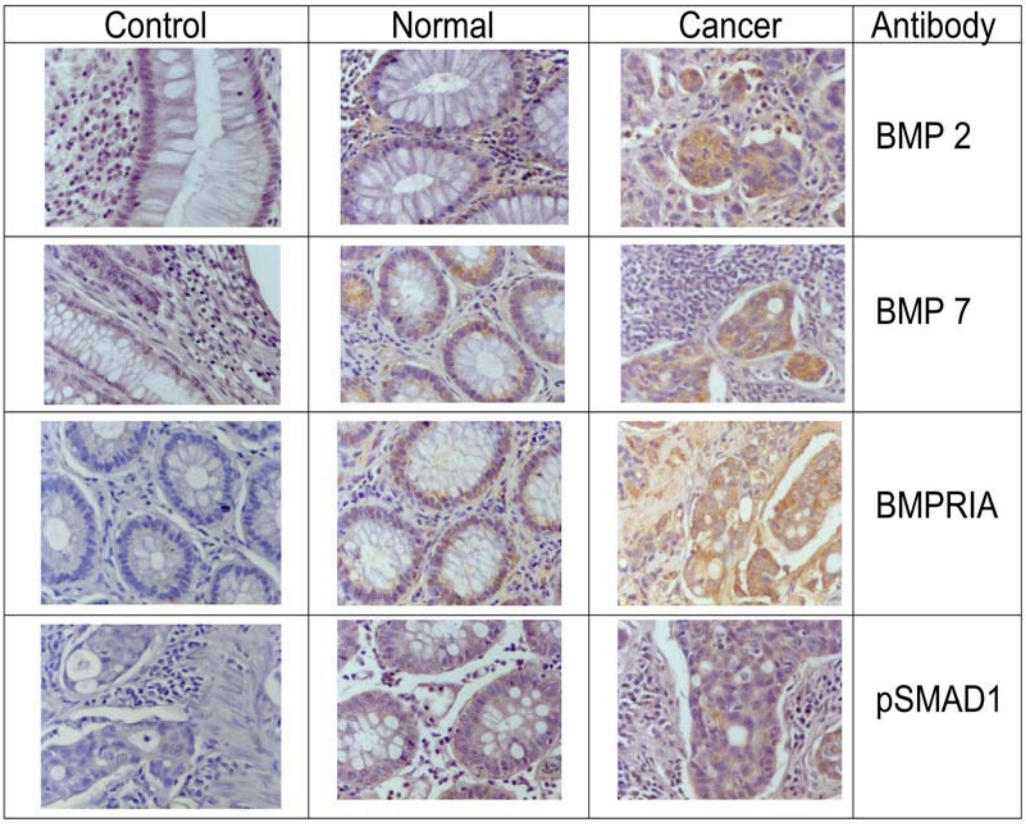



**Figure 3.8.** Representative pictures of immunohistochemistry (40X) on four paired human colon cancer specimens stained for BMP2, BMP7, BMPRIA, and pSMAD1. Controls represent stained samples without primary antibody. Intestinal hamartomatous polyps with and without mutations in BMPR1A were used as additional controls (not shown).

# Chapter 4:

# BMP-Induced Growth Suppression in Colon Cancer Cells is Mediated by $p21^{WAF1}$ Stabilization and Modulated by RAS/ERK

## Introduction

K-Ras, a member of the Ras family, commonly develops constitutively activating mutations at codon 12 or 13 as an early event during the progression of colorectal cancer in greater than 50% of colon cancers. (Rebollo, 1999; Derynck, 2003) EGF stimulation as well as oncogenic Ras can also modulate a target of the Ras pathway, SMAD1. SMAD1 can be phosphorylated at the linker region by Erk kinase, which slows or inhibits nuclear accumulation of activated SMAD1 in response to BMP ligands (Kretzschmar, 1997; Kretzschmar, 1997). It has also been shown in osteoblasts that BMP2 can activate the Ras signaling pathway through the SMAD4 pathway (Lai, 2002; Yue, 1999).

Multiple mutations in the cellular growth and progression pathways lead to the advancement of colorectal cancer. While many of these pathways have been deciphered, there is still much unknown about the role of the BMP signaling pathway in colorectal cancer. It is our hypothesis that inhibition of SMAD1 translocation to the nucleus in response to BMP by the RAS pathway results in the partial loss of growth inhibitory effects by BMP in colon cancer cells. This study is extremely relevant to the pathogenesis of colorectal cancer as approximately 50% of colorectal cancers have activating mutations in the RAS protein (Arber, 2000).

To this end, we utilized the colon cancer cell line FET. FET cells contain several alterations, including mutated *TP53* gene, overactive Wingless/Wnt and Ras/MAPK pathways (Gayet, 2001), but are non tumorigenic in nude mice, and

therefore are considered an early model of colon cancer. Previous work in our lab has shown that FET cells are strongly responsive to TGFβ. In this study, we aimed to answer the following questions: 1) Is BMP signaling intact and growth suppressive in FET colon cancer cells, and 2) if not, is the BMP pathway attenuated by the oncogenic Ras pathway in these cells? We found that FET cells are mildly responsive to BMP2 and that oncogenic Ras lessens the response to BMP2 in these cells.

**Results**

The Ras signaling pathway inhibits phospho SMAD1 from Entering the Nucleus in FET Colon Cancer Cells

Non-tumorigenic FET colon cancer cells have been described to contain intact TGFβ signaling, but the BMP pathway status is not known. Additionally, because FET colon cancer cells contain a mutated k-RAS gene, making it constitutively active, we examined if mitogenic pathways such as RAS/ERK, often activated in colon cancers, could negatively affect BMP signaling and thus negate its growth suppressive effects. We used pharmacological inhibition of ERK by the inhibitor PD98059, and inhibition of PI3K by the inhibitor LY294002, as well as directly inhibiting k-RAS by a transfected DN-kRAS. **Figure 4.1** shows that the use of these inhibitors or transfections were effective.

We first wanted to determine whether BMP2 or BMP7 mRNA was expressed in the FET colon cancer cell line and whether BMP2 treatment modified their expression**.** We performed semi-quantitative RT-PCR before and after BMP2 treatment and assessed the expression of BMP2 and BMP7, using GAPDH as a control. We found FET cells express both BMP2 and BMP7 and further treatment with BMP2 ligand did not modify this expression. **(Figure 4.2)**

To investigate whether the BMP pathway is perturbed in colon cancer cell lines, we first sought to examine whether the receptors were functional by treating cells with BMP2 and examining SMAD1 translocation to the nucleus by a luciferase reporter assay. Transcriptional activation due to BMP2 stimulation was determined with the use of the BMP-specific SMAD-induced luciferase reporter, BRE-Luc (a generous gift from Dr. Peter ten Dijke) (Korchynskyi, 2002). Transcriptional activation by BMP2 was three-fold increased over untreated cells **(Figure 4.3A)**. We investigated whether the Ras pathway could interfere with the BMP pathway. We treated FET cells with PD98059 (PD), a MEK1 specific inhibitor and combined we observed an even further increase in transactivation with BMP2 treatment. **(Figure 4.3A)**. We also transfected cells with a dominant negative k-RAS (DN k-Ras) (a generous gift from Dr. Rik Derynk, UCSF, San Francisco, CA) and found futher increases in SMAD transcriptional activity than to those obtained with PD drug **(Figure 4.3B)**. We further treated cells with LY294002, a PI3Kinase inhibitor, and found similar results as when MEK was

inhibited **(Figure 4.3C)**. We next utilized a phospho-SMAD1 specific antibody and performed immunofluorescence on the FET cells. Upon BMP2 stimulation, SMAD1 should become phosphorylated and translocate to the nucleus. BMP2 treatment induced an increase (2.5 fold) in nuclear phospho-SMAD1 immunofluorescence when compared to controls. (**Figure 4.4 A-E**). We inhibited the RAS pathway with PD98059 drug or DN k-RAS transfection and found that these improved nuclear phospho-SMAD1 alone and further improved with BMP2 treatment. We also obtained similar results when PI3Kinase (downstream of RAS) was inhibited with LY294002 drug.

The BMP2 pathway is growth suppressive in FET colon cancer cells and the Ras/Erk Signaling Pathway blocks BMP2-induced Growth Suppression

To determine if BMP2 treatment can modulate the proliferation of FET colon cancer cells, we treated cells with BMP2 and counted them at 4 and 6 days after treatment and performed the MTT metabolic assay as a surrogate for cell growth. We found BMP2 treatment to decrease the number of cells by an average of 25% as assessed by cell counting over several experiments. When we combined BMP2 treatment with MEK1 inhibition using the PD98059 inhibitor, we found a futher decrease in cell number of about 30% at day 4 and 60 % at day 6 when compared to treatment with PD98059 alone. There is a significant change in cell number between BMP2 treatment alone and BMP2 with PD98059 inhibitor at day

6 of cell treatment. We also utilized a dominant negative k-RAS to inhibit the Ras pathway, and found a significant change in cell number between BMP2 treatment in mock-transfected cells and BMP2 in DN k-RAS transfected cells at day 6 of cell treatment. We further examined inhibition with the PI3Kinase inhibitor LY294002 and found a significant change in cell growth at day 4 and day 6 of treatment when compared to BMP2 treatment alone. **(Figure 4.5 A-C)**

The MTT assay measure metabolic activity of cells, which is an indirect measure of cell growth. We found a 16% decrease in metabolic activity with BMP2 treatment at day 4 compared to a 50% significant decrease in metabolic activity with BMP2 and PD98059 treatment. Treatment with LY294002 and BMP2 resulted in a 40% significant decrease in metabolic activity over treatment with LY294002 alone at day 4. **(Figure 4.6 A-B)**

Growth Suppression in FET colon Cancer cells is partly due to an increase in p21 and is abrogated by the Ras Signaling Pathway

To examine the downstream effects of BMP2 stimulation on FET cells we examined if BMP2 treatment modulated expression of *PTEN* or *p21*. It has been shown in breast cancer that BMP2 treatment can increase levels of PTEN by inhibiting the association of PTEN with ubiquitination proteins and decreasing its degradation (Waite, 2003). We examined whether BMP2 could modulate PTEN levels in FET colon cancer cells at the transcription level by RT-PCR and luciferase

assay with a PTEN specific luciferase reporter, PTEN-luc; and at the protein level by Western blotting. We found no change in PTEN transcript when subjected to BMP2 treatment via RT-PCR and no change in PTEN expression via luciferase activity with PTEN-luc. Furthermore, we did not see a change in PTEN protein levels with BMP2 treatment. **(Figure 4.7 A-C)**

BMP2 treatment causes p21 upregulation in several cancer cell types including: breast, prostate, and gastric cancers (Pouliot, 2003; Brubaker, 2004; Ghosh-Choudhury, 2000; Ghosh-Choudhury, 2000; Wen, 2004; Yue, 1999; Yue, 1999). We tested the effect of BMP2 treatment on p21 transcription via the luciferase reporter assay utilizing a p21 specific reporter, pWWP-luc and on p21 RNA levels using RT-PCR. We found a 1.3 fold increase in p21 RNA levels using the luciferase assay and a slight increase in RNA levels with RT-PCR with BMP2 that was consistent when treated with PD98059 or LY294002. **(Figure 4.8 A-B)**

Utilizing western blotting, we found an increase in p21 protein levels with BMP2 (**Figure 4.8 C**). Because we only saw slight changes in RNA levels, we hypothesized this increase in p21 level was from inhibition of p21 degradation. To test this hypothesis, we stimulated p21 levels with BMP2 treatment, then treated cells with cyclohexamide (CHX) to inhibit new protein synthesis. Additionally, some cells were treated with BMP2 and then p21 protein stability monitored over 36 hours. We found less degradation of p21 in the cells treated with BMP2. We also performed this experiment in the presence of PD98059 and found less

degradation in cells treated with BMP2 and PD98059 than cells treated with just PD98059 or just BMP2. We also found less p21 degradation when the cells were transfected with DN k-RAS than mock vector. Additionally, cells treated with LY294002 showed a decrease in protein degradation levels compared to untreated cells, though we did not see a significant difference compared to cells treated with BMP2 alone **(Figure 4.9).**

BMP-SMAD signaling activates additional phospho-ERK above basal oncogenic RAS-induced activation

It has been demonstrated in osteoblasts that BMP2 can stimulate RAS/MAPK activity (Lai & Cheng, 2002). We assessed if BMP2 treatment can stimulate phospho-ERK activity in FET cells. BMP2 treatment activated ERK (in conjunction with increased nuclear phospho-SMAD1), by 10 minutes after BMP2 treatment that persisted through 120 minutes. The phospho-ERK activation is in addition to the constitutive levels stimulated by oncogenic RAS in these cells (**Figure 4.10**), as FET colon cancer cells contain a mutated K-RAS gene (codon 12 mutation: GGT > GCT) (Kopreski et al., 2000), making K-RAS constitutively active. BMP2 activation of phospho-ERK may act as a "brake" on BMP-induced growth suppression by regulating or limiting this response in the correct setting. Treatment of cells with the ERK inhibitor PD98059 abolished both constitutive

phospho-ERK activation and BMP2-induced phospho-ERK activation in these cells (**Figure 4.10**).

**Discussion**

The BMP2 pathway is intact in FET cells and FET cells are growth inhibited by BMP2. FET cells contain oncogenic RAS, which helps to drive their growth. We inhibited the effects of oncogenic RAS in FET cells by using chemical inhibitors and transfection of a dominant negative k-RAS. At all levels of RAS pathway inhibition that we performed, we found increased growth suppression and more phospho-SMAD1 signaling to the nucleus with BMP2 treatment. Therefore, the RAS signaling pathway is capable of dulling the effect of BMP2 in FET colon cancer cells. Thus, we observe an additional new way RAS is able to modulate the growth of FET colon cancer cells (1) activating mutations in RAS stimulate growth (Grady & Markowitz, 2002) and (2) RAS is able to dull or inhibit BMP2's effect on FET colon cancer cells by inhibiting or limiting the amounts of phospho-SMAD1 translocating to the nucleus and preventing p21 degradation.

In conclusion, we have shown that when the RAS pathway is inhibited by chemical inhibitors and a dominant negative k-RAS construct, BMP2 is able to exert a stronger growth suppressive effect, in part by further stabilizing p21 protein in the cell. To the best of our knowledge, this is the first time it has been shown that inhibition of the RAS pathway can lead to enhanced growth suppression in FET

colon cancer cells and that RAS inhibition can lead to further stability of p21 in FET colon cancer cells.

**Acknowledgements**

A majority of the content of Chapter 4 has been submitted for publication (**Beck SE**, Jung B, Del Rosario E, Gomez J, and Carethers J. "Activated RAS modulates Bone Morphogenetic Protein (BMP)-induced Growth Suppression" *(In Submission)*Stayce Beck was the primary researcher and author for this chapter. John Carethers supervised and directed the research in this chapter. Eunice del Rosario assisted in the cell counting and MTT experiments. Barbara Jung and Jessica Gomez assisted in the p21 experiments. Digital microscopic examination was performed at the UCSD Cancer Center Digital Imaging Shared Resource.

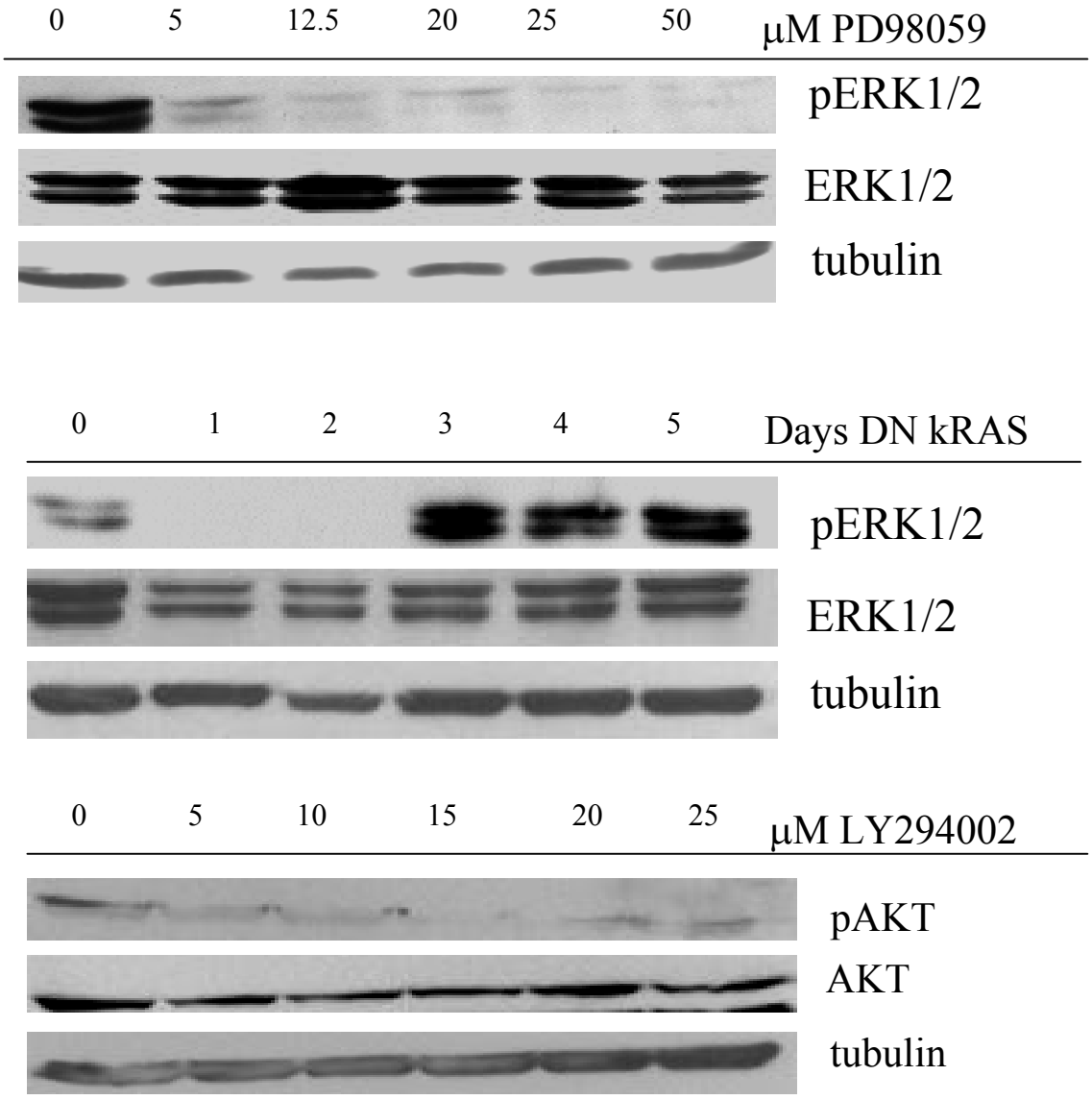



**Figure 4.1.** Western Blots (i) 10 min treatment of FET cells with increasing amounts of PD98059 and (ii) DN k-RAS showing inhibition of phospho ERK1/2 and (iii) 30 min. of increasing amounts of LY294002 showing inhibition of phospho AKT

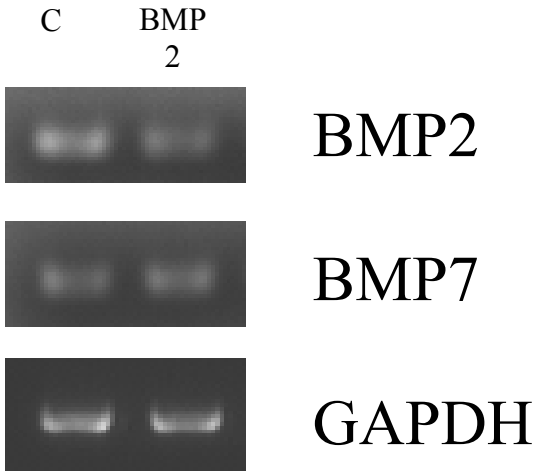



**Figure 4.2.** Reverse Transcriptase PCR of FET cells for BMP2 or BMP7. Cells were untreated or treated with 100 ng/ml of BMP2; GAPDH was used as a control.

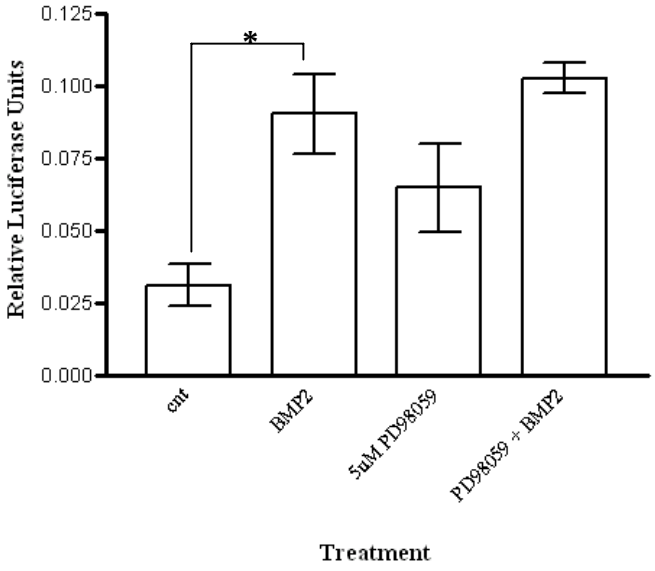



**Figure 4.3(*A*).** Luciferase reporter assay utilizing BRE-Luc and treating cells with 100 ng/ml of BMP2 and with and without inhibitors of the Ras Pathway (*$p<0.05$).

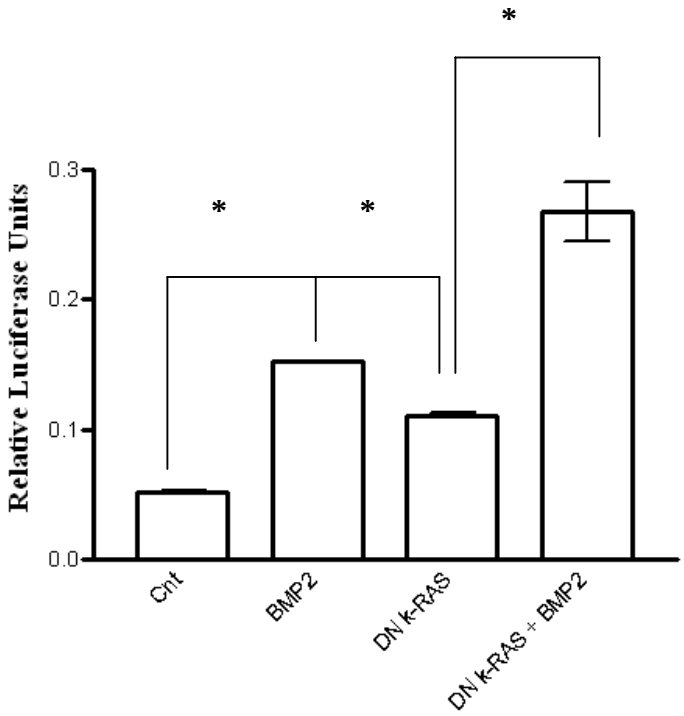



**Figure 4.3(*B*).** Luciferase reporter assay utilizing BRE-LUC and treating cells with 100 ng/ml of BMP2 and with and without inhibitors of the Ras Pathway (*$p<0.05$).

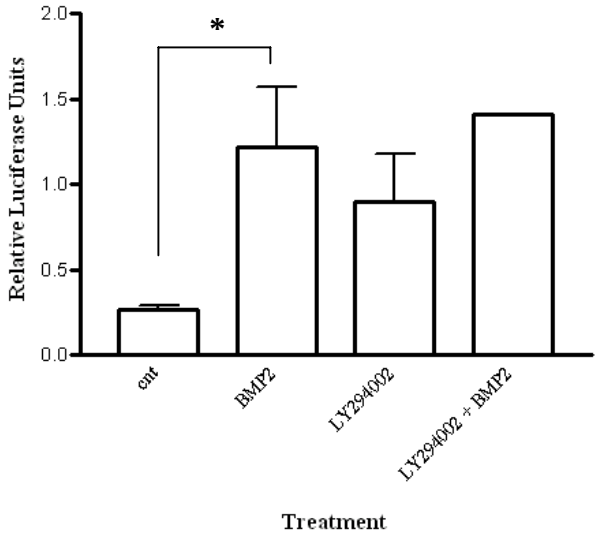



**Figure 4.3(*C*).** Luciferase reporter assay utilizing BRE-LUC and treating cells with 100 ng/ml of BMP2 and with and without inhibitors of the Ras Pathway (*$p<0.05$).

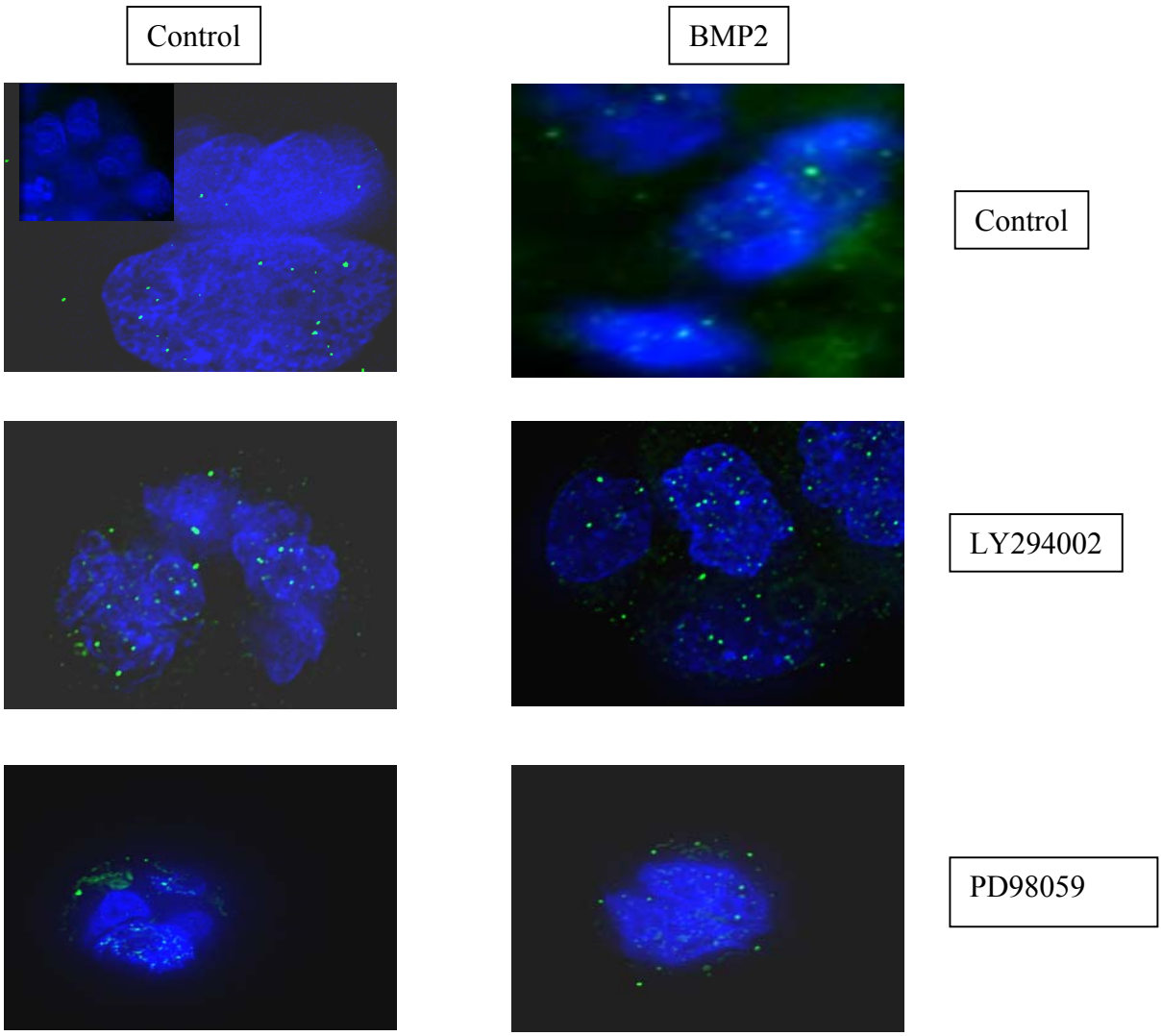



**Figure 4.4 (*A-E*).** Immunofluorescence using phospho-SMAD1 antibody (green), the nuclei are stained with DAPI (blue). (A) FET cells with (B) PD98059, (C) LY294002, and (D) pcDNA3.0 and (E) DN k-RAS.

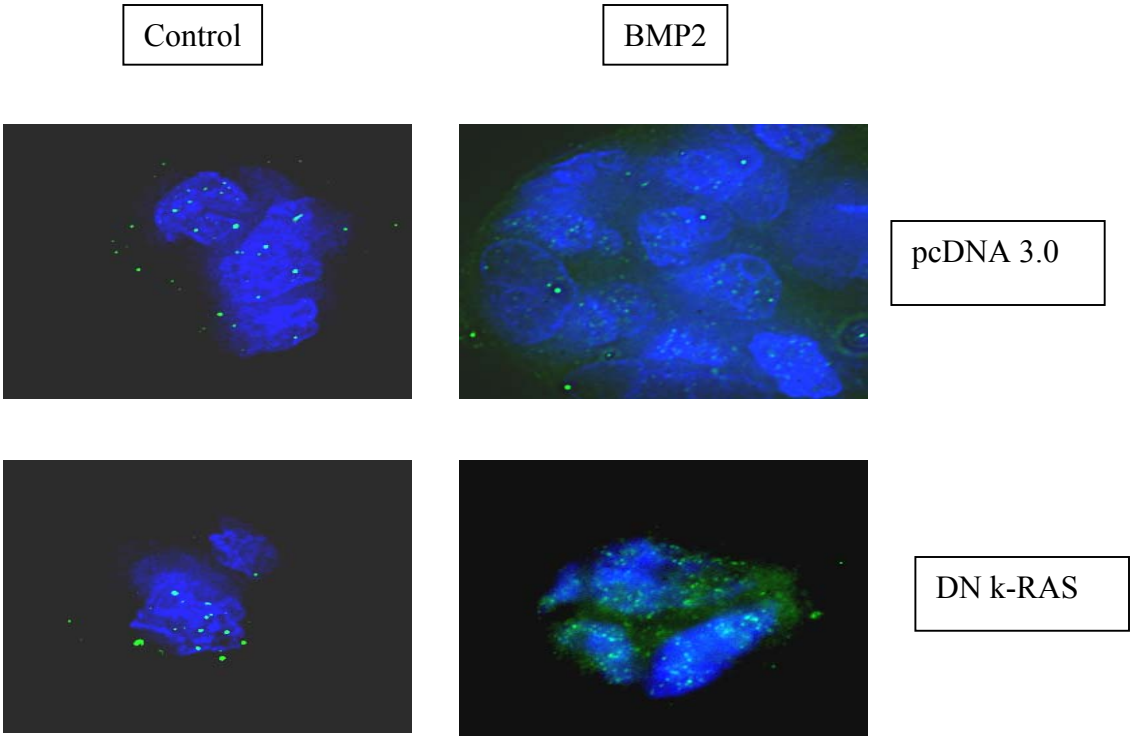



**Figure 4.4 (*A*-*E*).** Immunofluorescence using phospho-SMAD1 antibody (green), the nuclei are stained with DAPI (blue). (A) FET cells with (B) PD98059, (C) LY294002, and (D) pcDNA3.0 and (E) DN k-RAS.

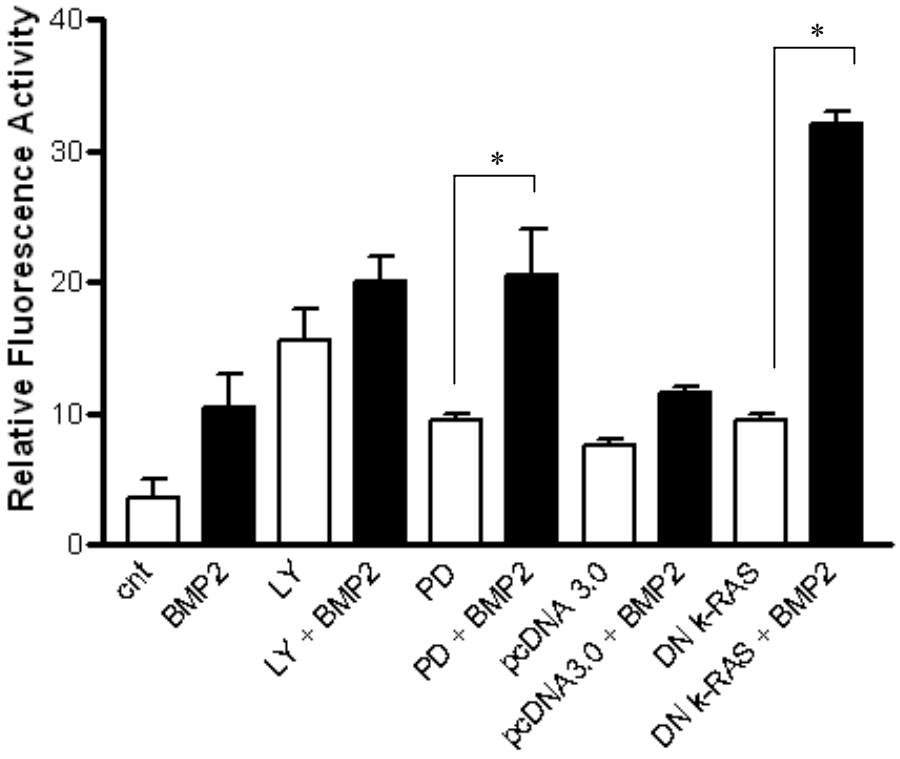



**Figure 4.4 (*F*).** (F) Summary graph of nuclear immunofluorescence data for FET cells probed with phospho-SMAD1 (*$p<0.05$). These experiments were repeated three times and this is one representative experiment.

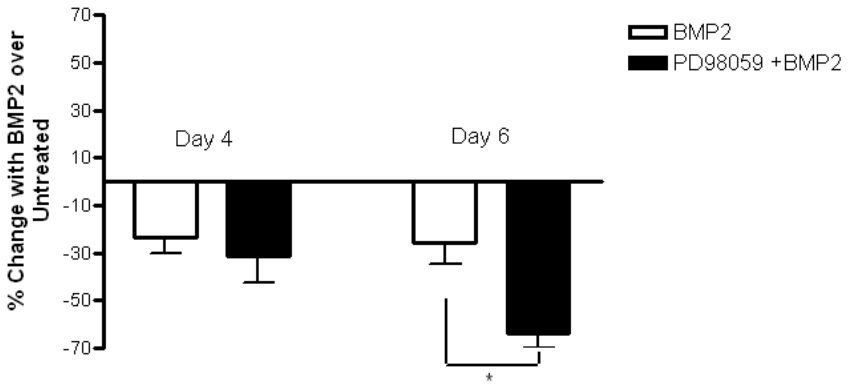



**Figure 4.5 (*A*).** Cell growth assay on FET cells treated with 100 ng/ml of BMP2 and with or without PD98059. Results are expressed as percent change in growth with BMP2 treatment compared to untreated controls or just treated with inhibitor (ie. PD98059 with BMP2 change compared with just PD98059)

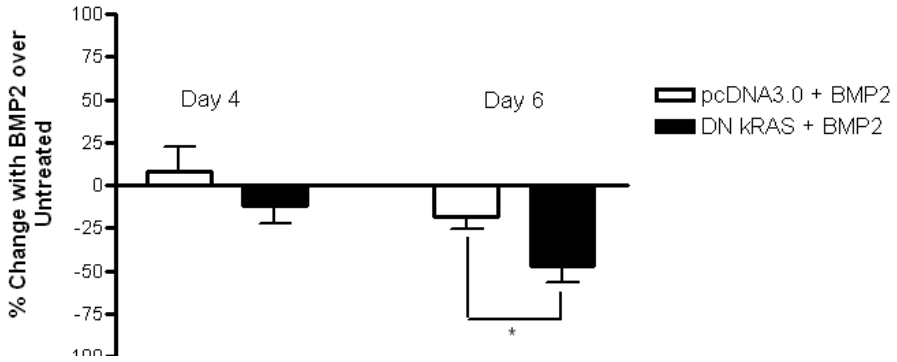



**Figure 4.5 (*B*).** Cell growth assay on FET cells treated with 100 ng/ml of BMP2 and with or without DN kRAS. Results are expressed as percent change in growth with BMP2 treatment compared to untreated controls or just treated with inhibitor (ie. DN kRAS with BMP2 change compared with just DN kRAS)

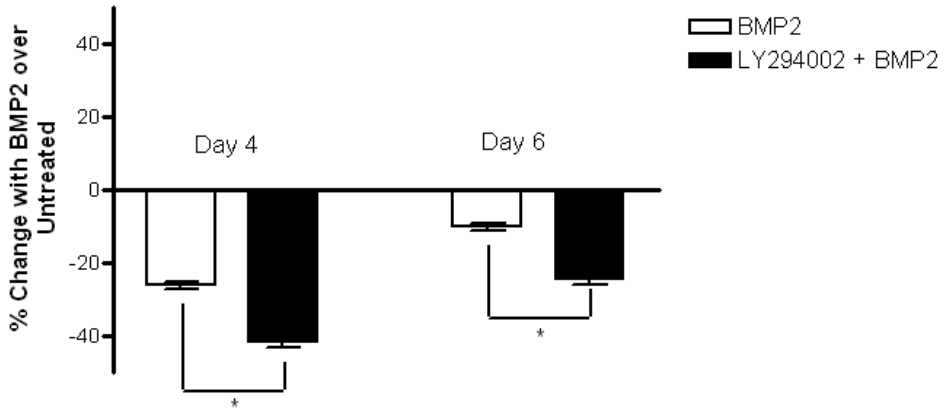



**Figure 4.5 (*C*).** Cell growth assay on FET cells treated with 100 ng/ml of BMP2 and with or without LY294002. Results are expressed as percent change in growth with BMP2 treatment compared to untreated controls or just treated with inhibitor (ie. LY294002 with BMP2 change compared with just LY294002)

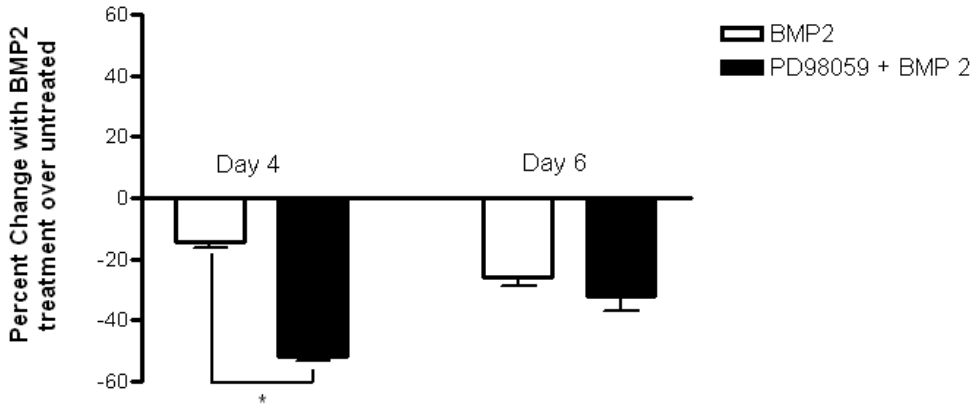



**Figure 4.6 (*A*).** MTT assay data on FET cells with 100 ng/ml of BMP2 and with or without PD98059.

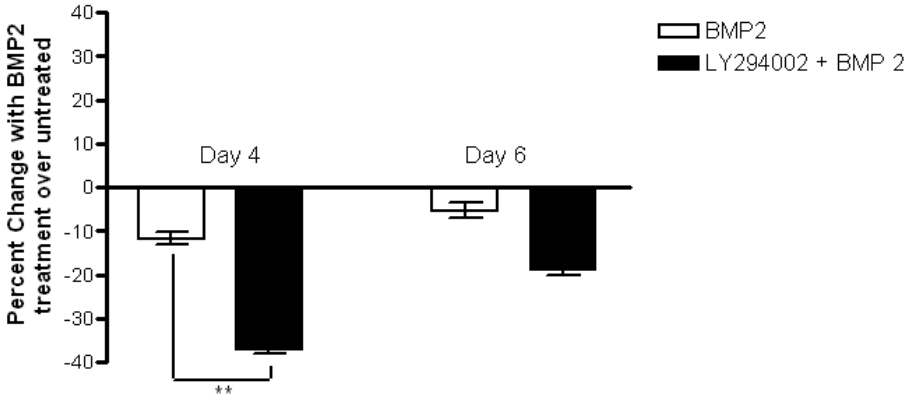



**Figure 4.6 (*B*).** MTT assay data on FET cells with 100 ng/ml of BMP2 and with or without LY294002.

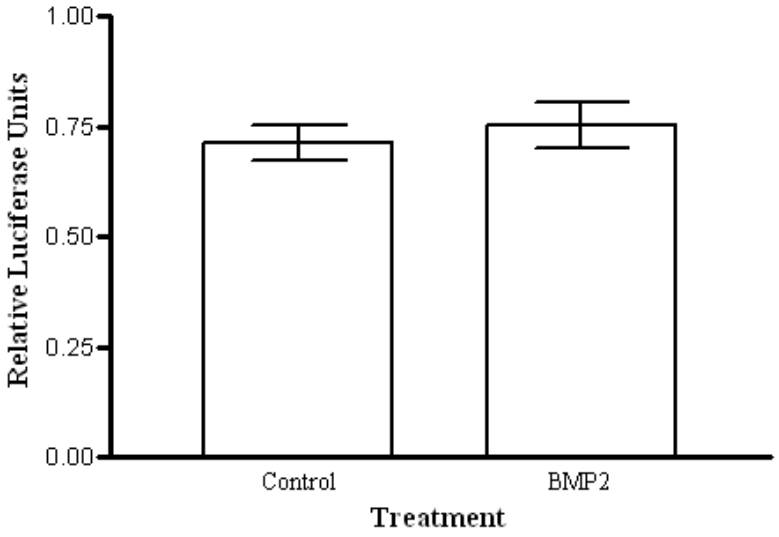



**Figure 4.7 (A).** PTEN expression in FET Colon cancer cells with 100 ng/ml of BMP2 PTEN-luc luciferase assay.

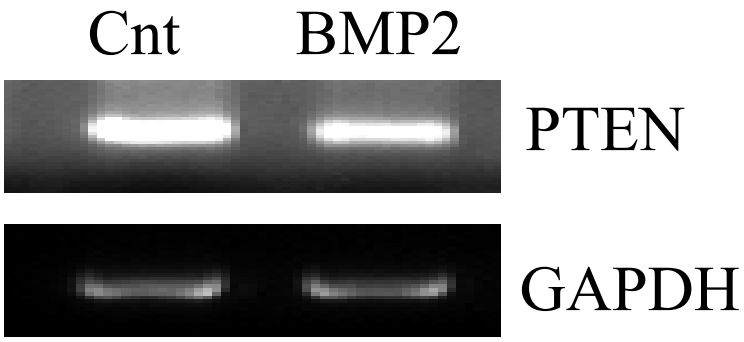



**Figure 4.7 (*B*).** PTEN mRNA expression in FET Colon cancer cells with 100 ng/ml of BMP2 via semi-quantitative RT- PCR.

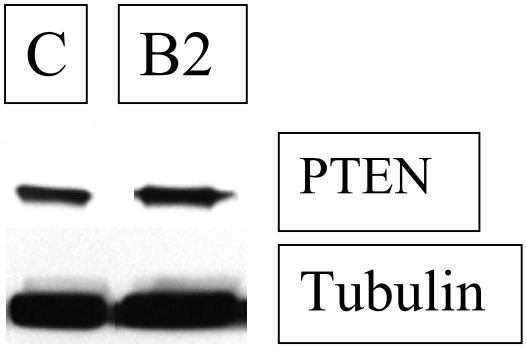



**Figure 4.7 (*C*).** PTEN expression in FET Colon cancer cells with 100 ng/ml of BMP2 PTEN western blot.

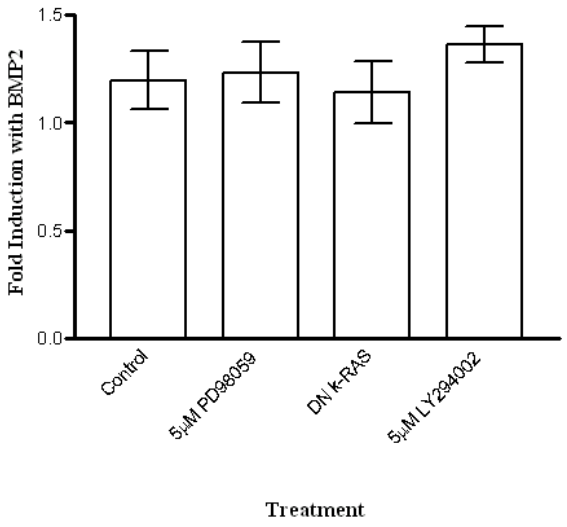



**Figure 4.8 (*A*).** p21 transactivation in FET Colon cancer cells with 100 ng/ml of BMP2 and inhibitors in this pWWP-luc luciferase assay.

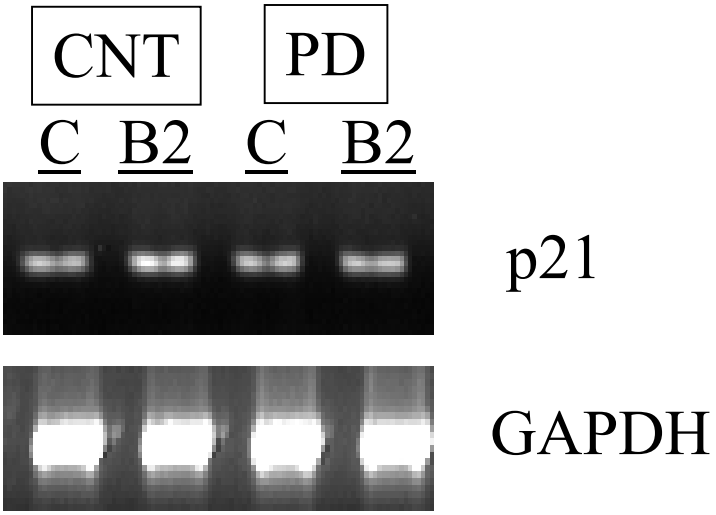



**Figure 4.8 (*B*).** p21 expression in FET Colon cancer cells with 100 ng/ml of BMP2 and inhibitors p21 RT-PCR

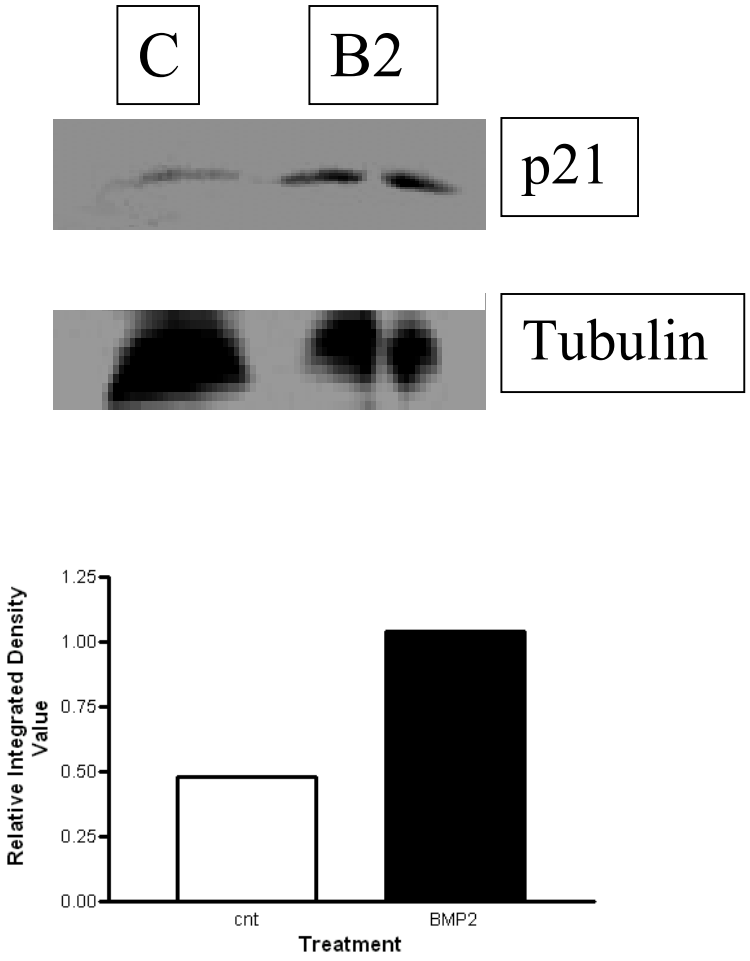



**Figure 4.8 (*C*).** Western Blot of p21 protein expression in FET Colon cancer cells with 100 ng/ml of BMP2 and densitometry.

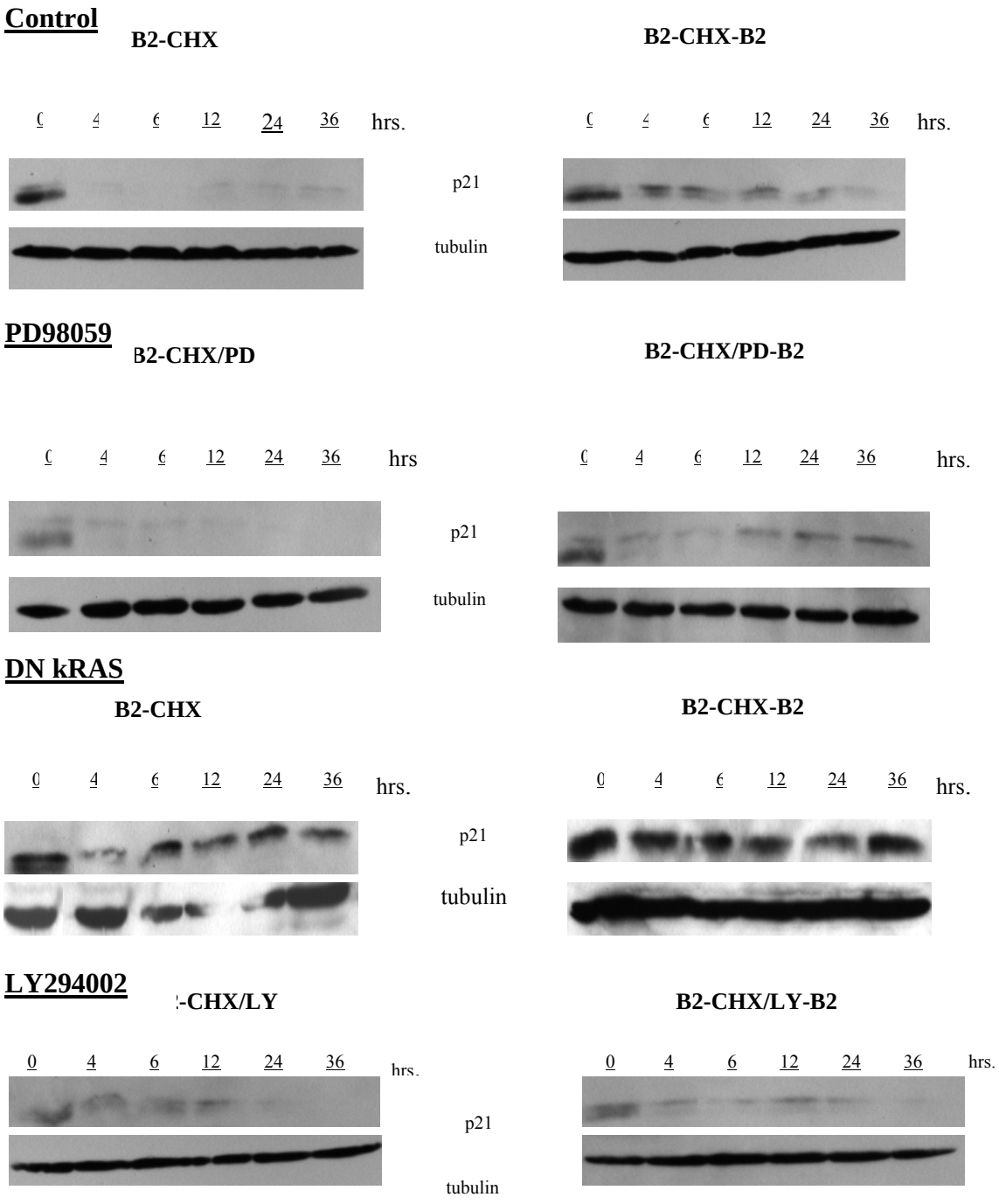



**Figure 4.9.** p21 protein expression after 12 hrs. stimulation with BMP2, addition of Cyclohexamide (CHX), then with or without BMP2 and inhibitors for various time points.

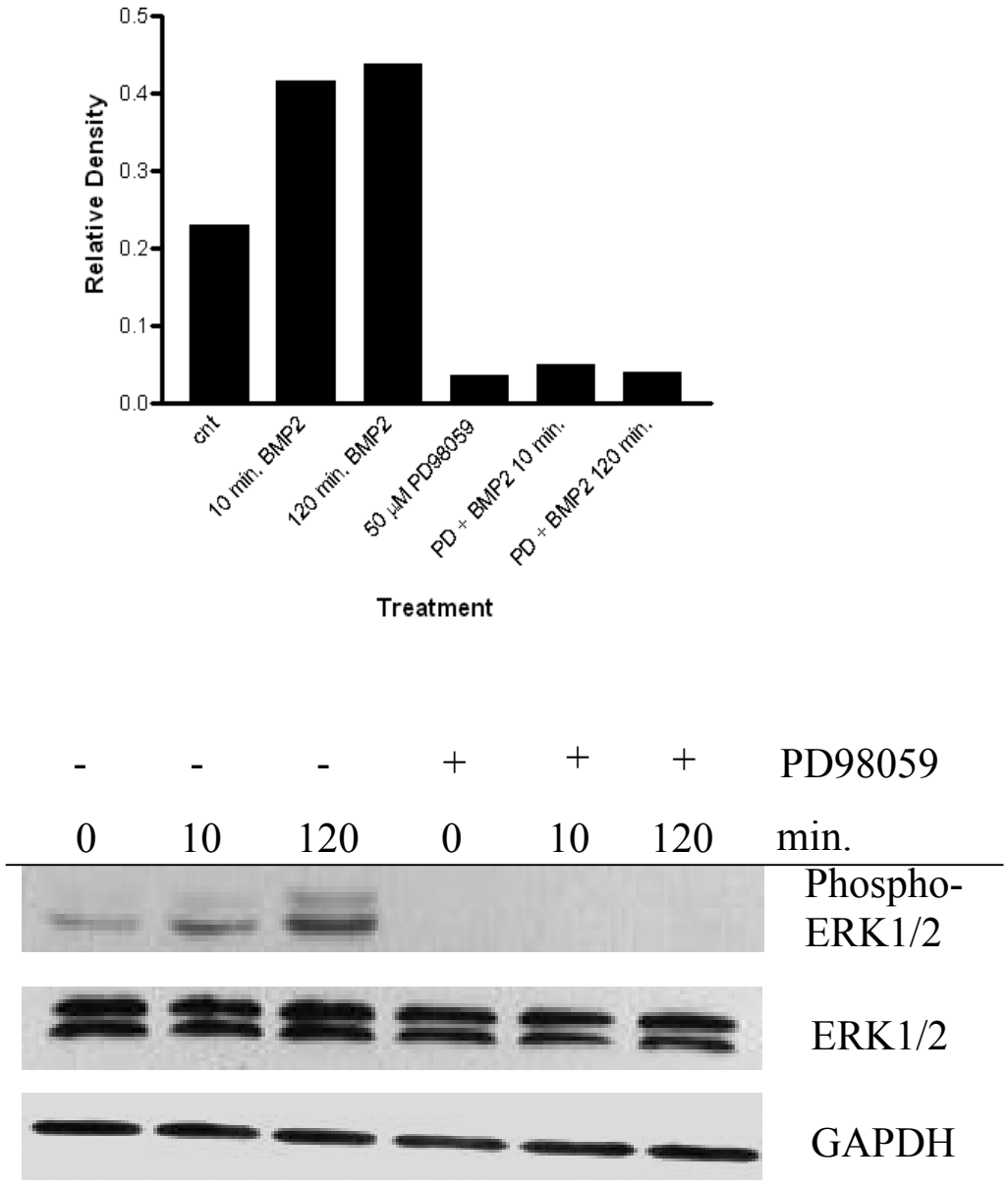



**Figure 4.10.** (a) Densometry of phospho-ERK compared with GAPDH, (b) Western Blot of FET cells treated for various time points with 100 ng/ml of BMP2 and 5μM PD98059 where indicated.

# Chapter 5:

# BMP Reduces PTEN levels through the RAS/ERK Pathway in SMAD4-null Colon Cancer Cells

**Introduction**

Classically, BMP ligand utilizes the SMAD1/SMAD4 signaling pathway to transmit its signal to the nucleus, but SMAD4 often becomes mutated at later stages of colon cancer progressiont. This begs the question does BMP ligand utilize SMAD4-independent pathways to modulate the cell? Additionally, JP, an autosomal dominant gastrointestinal hamartomatous polyposis syndrome that increases the afflicted patient's risk for developing colon cancer ~12-fold, has germline mutations in the tumor suppressor *SMAD4* and *BMPRIA* (Howe, 1998; Zhou, 2001). Additionally, a small percentage of JP kindreds have also shown germline mutations in *PTEN* (Huang, 2000). Therefore, a very important question that is still not understood is whether the BMP signaling pathway interacts with the PTEN signaling pathway as both cause JP syndrome. Eng et al. found that BMP treatment increased PTEN protein levels by decreasing the association of PTEN with ubiquitin degradation proteins (Waite, 2003). Qiao et al. used a Cre-loxP approach to disrupt the SMAD4 gene in skin to study epidermal tumorigenesis. They showed that the absence of Smad4 blocked TGFβ and BMP SMAD signaling and that the mice developed malignant skin tumors. Interestingly, they found that tumorigenesis is accompanied by inactivation of PTEN, and subsequent activation of AKT (Qiao, 2005). These initial studies indicate there is likely a connection between the BMP pathway and the PTEN pathway.

Here we aimed to determine whether SMAD4-null SW480 cells respond to BMP ligand, and whether there is any interaction between the BMP and PTEN signaling pathways. Unexpectedly, we found that cells that are SMAD4-null still

respond to BMP ligand treatment (Beck et al., 2006). We found that while initial growth is suppressed by BMP in SMAD4-null colon cancer cells, over a longer treatment time, BMP becomes growth proliferative in these cells. This switch appears to be in part modulated by PTEN as BMP treatment decreases PTEN mRNA levels at the transcriptional level and appears modulated by the RAS/ERK pathway.

## Results

### BMP-induced specific transcription is intact in SMAD4-null colon cancer cells

Transcriptional activation due to BMP2 or BMP7 stimulation was determined with the use of the BMP-specific SMAD-induced luciferase reporter, BRE-Luc (a generous gift from Dr. Peter ten Dijke) (Korchynskyi, 2002). Non-transfected and mock-transfected *SMAD4*-null SW480 cells did not exhibit increased transcriptional activity when treated with BMP2 or BMP7 over untreated controls **(Figure 5.1)**. To determine the effect of reconstituting the SMAD4 pathway on BMP signaling, we transfected *SMAD4* into SW480 cells. The *SMAD4*-transfected SW480 cells showed a modest increase in BRE-Luc activation when treated with ligand over control; however, there was strong basal transcriptional activation of SMAD signaling in the absence of exogenous BMP, suggesting an autocrine system in these cells with stimulation of the BMP receptors **(Figure 5.1)**. When the *SMAD4*-transfected SW480 cells were treated with 200 ng/mL noggin, a BMP-specific inhibitor, BMP-induced transcriptional activity in these cells was reversed, confirming the high endogenous BMP activity. To further confirm the endogenous production of BMP2

and BMP7, we treated SW480 cells with BMP2, BMP7 and noggin for 1, 6, 24 and 48 hours and then performed semi-quantitative reverse transcriptase PCR on the SW480 cells. SW480 cells produced both endogenous BMP2 and endogenous BMP7 at the transcriptional level **(Figure 5.2)**. We did not observe transcriptional changes in endogenous BMP2 and BMP7 expression with ligand or inhibitor (noggin) treatment.

BMP ligand is growth suppressive in SMAD4-null SW480 cells at early time points

Cell growth was indirectly evaluated by MTT assay and directly by cell counting. **(Figure D3)** Although transcriptional activity was unchanged by exogenous BMP treatment, SW480 cells showed significant growth suppression when treated with 100 ng/ml of BMP2 or BMP7 for 48 hours as assessed by MTT assay **(Figure 5.3)**. Direct cell counting of the SW480 cells after BMP treatment also showed similar decreased growth of BMP-treated cells compared to untreated cells. We observed inhibition after 2 days of growth in the SW480 cells with either BMP2 or BMP7 ligand **(Figure 5.4)**.

Dominant negative BMPR1A reverses transcriptional activity and growth inhibitory effects of BMP ligands in SMAD4-null SW480 cells

We transfected SW480 cells with a *BMPRIA* dominant negative construct (DN-BMPR1A) and compared the results with a transfected BMPRIA constitutively active (CA BMPR1A) vector or mock vector (He et al., 2004). Post transfection, we

treated the cells with BMP2 or BMP7, and assayed transcription via the BRE-luciferase assay.

In SMAD4-null SW480 cells, DN BMPR1A transfection demonstrated no difference in transcriptional activity over mock transfection **(Figure 5.5)**. However, when DN BMPR1A was co-transfected with SMAD4, BMP2-induced transcriptional activity was reduced by 50% compared to SMAD4 transfection alone, although transcriptional activity was not reduced to the levels seen with noggin treatment. Thus, DN BMPR1A transfection reduces BMP-SMAD mediated transcription in our cell model.

Additionally, DN BMPR1A reversed the BMP-induced growth suppressive effects in our cell model. In SMAD4-null SW480 cells, the presence of DN BMPR1A completely reversed BMP2-induced growth suppression to levels similar to that treated with the BMP inhibitor noggin **(Figure 5.6)**. The combination of DNBMPRIA and noggin allowed cells to be even more proliferative over control. This combination removes endogenous ligand and prevents receptor activation by BMPs. Thus, we show reversal of BMP-induced growth suppression when signaling through BMPR1A is impaired, indicating that this receptor is a gateway for growth suppression.

<u>BMP2 decreases wound closure and invasion by SW480 cells</u>

We performed a wound closure assay on SW480 cells treated with BMP2, BMP7, and noggin. We found cells that were left untreated were able to close the wound gap, while BMP2 inhibited wound closure in SW480 cells. BMP7 also

inhibited the wound closure by SW480 cells but to a lesser extent than BMP2 did. Noggin, a BMP ligand inhibitor, did not inhibit wound closure by SW480 cells to any significant extent **(Figure 5.7)**. Using an invasion assay by Chemicon (Temecula, CA), we were able to show that BMP2 treatment inhibited the ability of SW480 cells to invade through an extracellular matrix (ECM) after 24 hours of treatment, while noggin did not **(Figure 5.8)**.

BMP causes a switch from being growth suppressive to growth proliferative with prolonged exposure/treatment

Initially, BMP treatment resulted in a decrease in cell growth in SMAD4-null SW480 cells after 24 to 48 hours **(Figures 5.3 and 5.4)**. Surprisingly, when we extend the length of time that SW480 cells were treated with BMP from 2 days out to 6 days, the SMAD4-null cells switched at about 4 days to growth proliferative. At day 6, there are significantly more cells when treated with BMP than when left untreated as assayed by direct cell counting **(Figure 5.9)**.

BMP treatment decreases PTEN levels over time, and correlates with changes in the growth pattern

One possible mechanism for how BMP switches from being growth suppressive to proliferative over time is that BMP ligand downregulates some growth suppressive genes. We examined several candidate growth suppressive genes and found that BMP2 ligand reduced levels of PTEN at the transcription, mRNA and protein levels. This decrease in PTEN mRNA and transcription occurred after 36

hours of BMP2 treatment **(Figures 5.10 and 5.11)**, while the decrease in PTEN protein levels occurred after 84 hours of BMP2 treatment **(Figure 5.12 and 5.13)**. Additionally, we noted a corresponding increase in phospho-AKT levels with the decrease in PTEN levels, implying that this BMP induced PTEN decrease had a functional effect on the PI3K pathway. This decrease in the PTEN tumor suppressor corresponds with a switch from growth suppression to growth proliferation that is significant after 6 days of BMP2 treatment. Additionally, treatment with noggin, a BMP ligand inhibitor does not result in a decrease of PTEN levels **(Figure 5.14)**.

BMP-induced PTEN suppression and growth proliferation are mediated by the RAS/ERK pathway

SW480 cells were treated with PD98059, a MEK1/2 kinase inhibitor, and treated with BMP2. We show via semi-quantitative RTPCR, PTEN luciferase assay and Western blot that with inhibition of the ERK kinase BMP-induced PTEN suppression is abolished **(Figure 5.14, 5.15, and 5.16)**. Correspondingly, we did not see an increase in phospho-AKT levels with MEK inhibition, and BMP2 treatment failed to suppress PTEN levels with MEK inhibition **(Figure 5.17)**. Additionally, we also performed cell counting assays with PD98059 and BMP2 treatment and showed that the cells no longer become growth proliferative with BMP2 treatment for six days **(Figure 5.18)**. These results suggest RAS/ERK influences BMP-induced PTEN regulation allowing BMP2 to switch from growth suppressive to growth proliferative by decreasing PTEN levels and increasing in phospho-AKT levels.

## Discussion

SW480 is a *SMAD4*-null cell line that surprisingly responds to BMP2 and BMP7 treatment by utilizing SMAD4 independent pathways. We show that these epithelial colon cancer cells produce endogenous BMP2 and BMP7 ligand, which suggests autocrine capabilities in addition to paracrine function by BMP ligands produced by fibroblasts and acting on the epithelial cells. Additionally we found that exogenous BMP2 may slow growth of BMP-sensitive colon cancer cells at early time points, but over time, BMP2 switched from growth suppressive to growth proliferative. Taken together, these findings indicate that BMP can induce SMAD4-independent growth effects in SW480 cells by a yet, uncharacterized pathway.

One mechanism for how BMP2 ligand induces a switch from growth inhibition to proliferation in these SMAD4-null SW480 cells is by decreasing the levels of the tumor suppressor PTEN. Long-term BMP2 treatment (36 hours for the mRNA and 84 hours for the protein resulted in decreased protein levels with resultant increases in phospho-AKT levels, paralleling the observed growth proliferation. Our results suggest that BMP2 induces these changes in PTEN levels and is modulated by the RAS/ERK pathway, as inhibition of this pathway abates BMP2-induced PTEN suppression and the resulting growth suppression with BMP2 treatment.

## Acknowledgements

The contents of Chapter 5 will be submitted for publication in part or in full. Stayce Beck was the primary researcher and author for this chapter. John Carethers supervised and directed the research that forms the basis of this chapter.

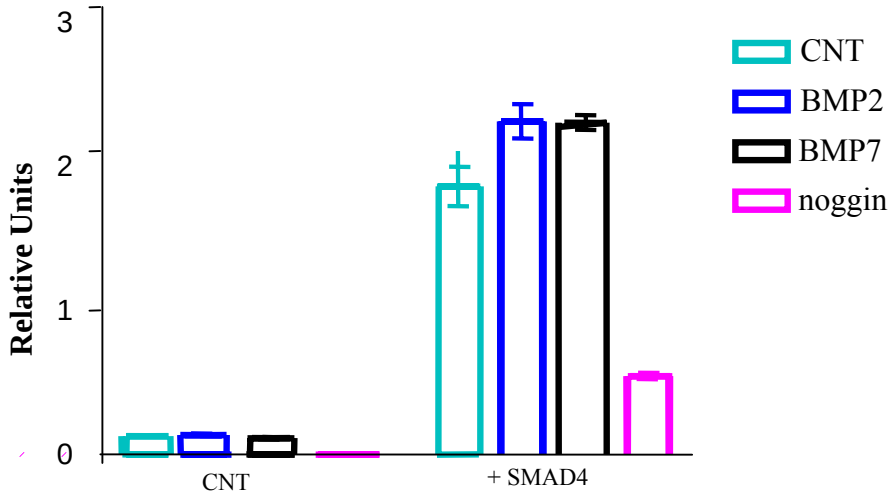



**Figure 5.1.** BRE-luc SMAD induced transcriptional activity in SMAD4 null cells. Cells were transfected with either mock vector or SMAD4 and treated with 50 ng/ml of BMP2 or BMP7 or 200 ng/ml of Noggin.

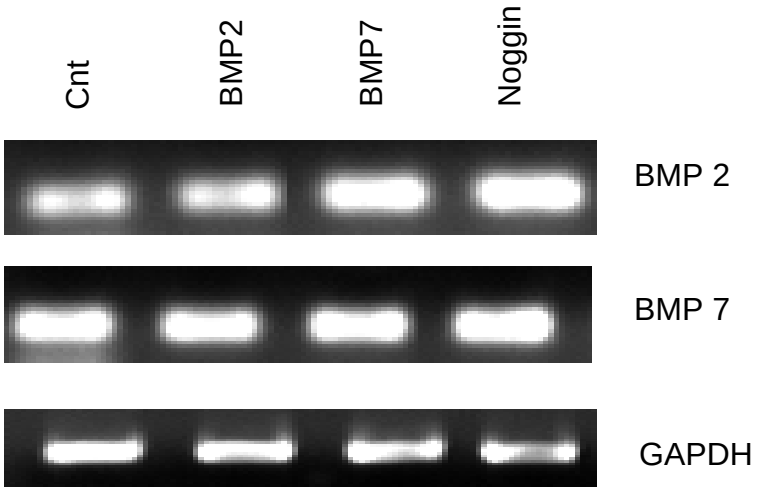



**Figure 5.2**. Semi-quantitative reverse transcriptase PCR of SW480 cells for basal (endogenous) BMP2 and BMP7 expression and after treatment with BMP2, BMP7, or noggin. GAPDH was used as a control.

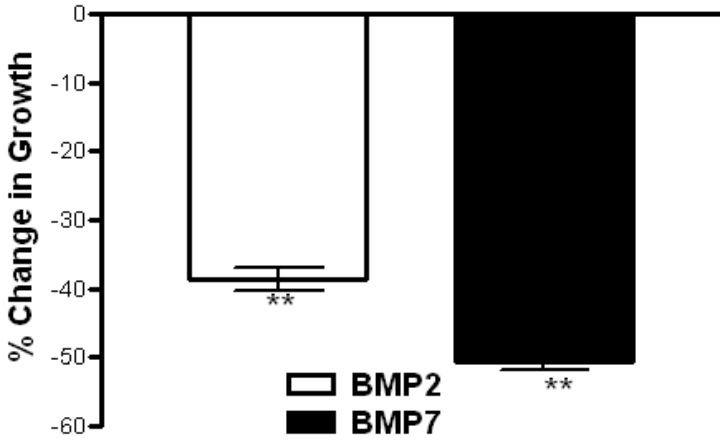



**Figure 5.3**. Effect of BMP2 or BMP7 treatment on cell growth measured indirectly by the MTT assay in SW480 cells after 2 days of BMP2 and BMP7 treatment. Graph is expressed as percent change in growth with treatment from untreated (**$p<0.01$).

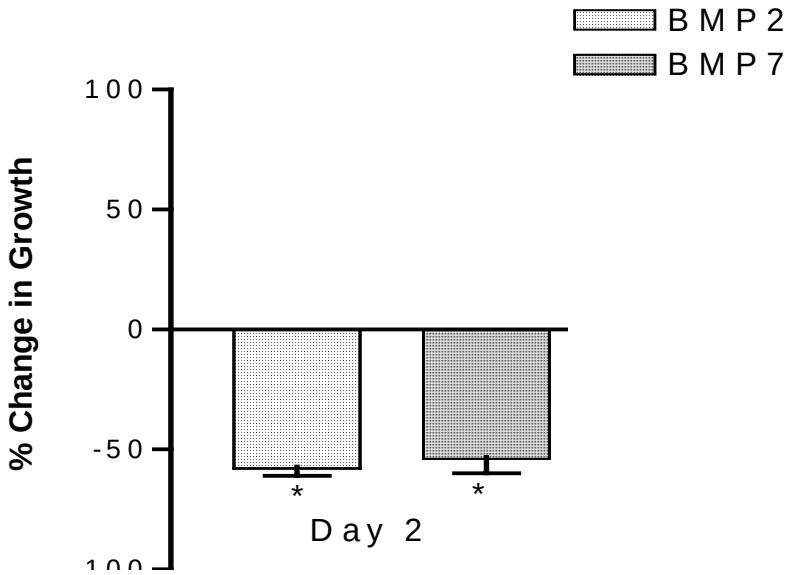



**Figure 5.4**. Effect of BMP2 or BMP7 treatment on cell growth as assessed by cell counting in SW480 cells after 2 days of BMP2 and BMP7 treatment (*$p<0.05$).

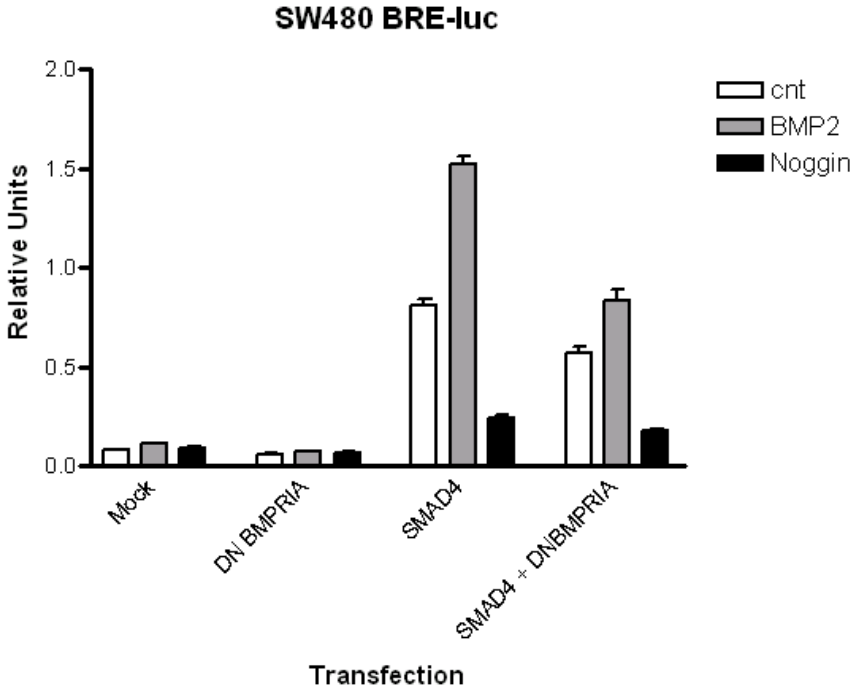



**Figure 5.5**. Relative amount of SMAD-induced transcriptional activity after BMP ligand treatment, and the effect of dominant negative (DN) BMPR1A transfection in *SMAD4*-null SW480 cells. DN BMPR1A reduces endogenous (control, cnt) and BMP2-induced SMAD transcriptional activity when co-transfected with *SMAD4*.

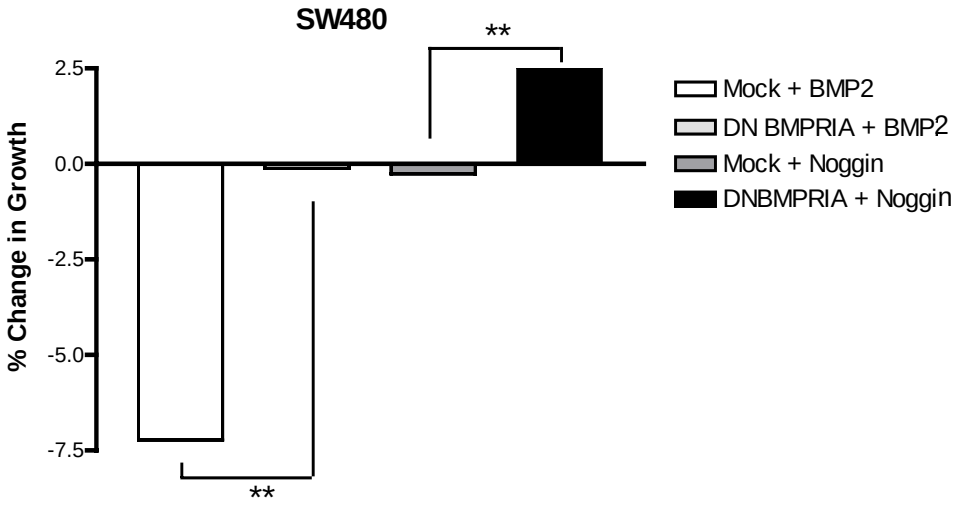
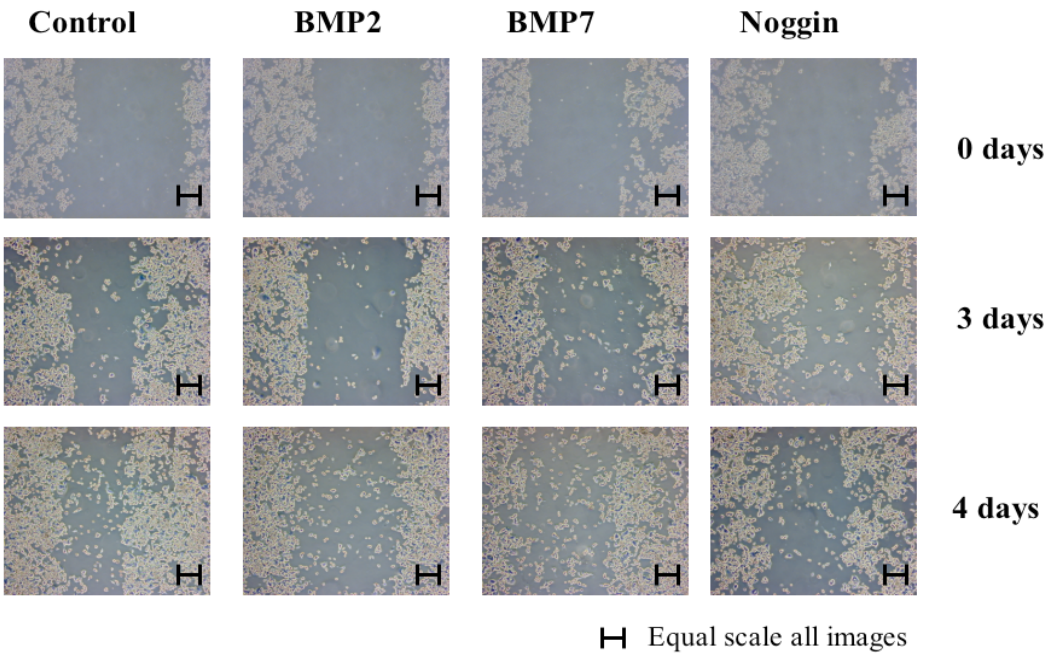



**Figure 5.6**. Dominant negative (DN) BMPR1A transfection reverses BMP-induced growth suppression as assessed by MTT assay in SW480 cells. Two-factor with replications ANOVA was used to determine p-values ( **$p<0.01$).



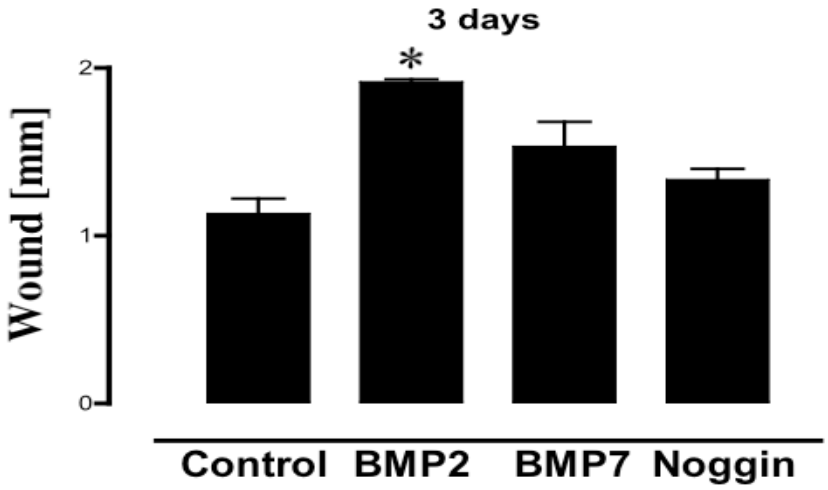




**Figure 5.7**. Wound closure assay of SW480 cells when untreated (control), and treated with BMP2, BMP7, or Noggin. (*A*) Representative pictures are shown here for day 0, day 3, and day 4. (*B*) Bar graph of SW480 wound closure at day 3 (*$p<0.05$).

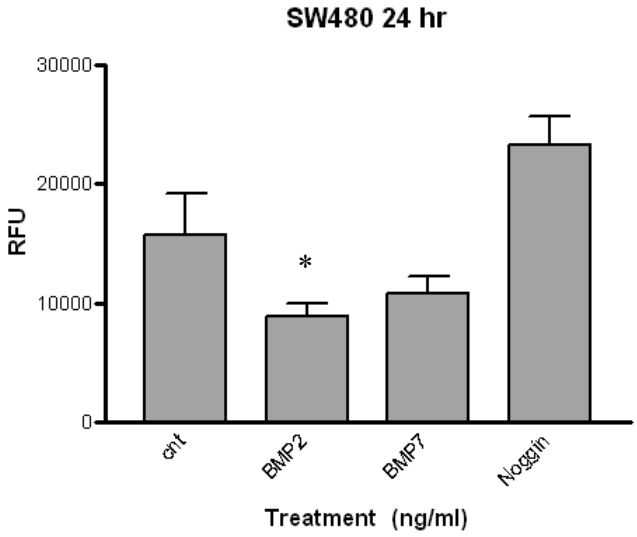



**Figure 5.8**. Invasion assay of SW480 cells when untreated (control), and treated with BMP2, BMP7, or Noggin for 24 hours (*$p<0.05$).

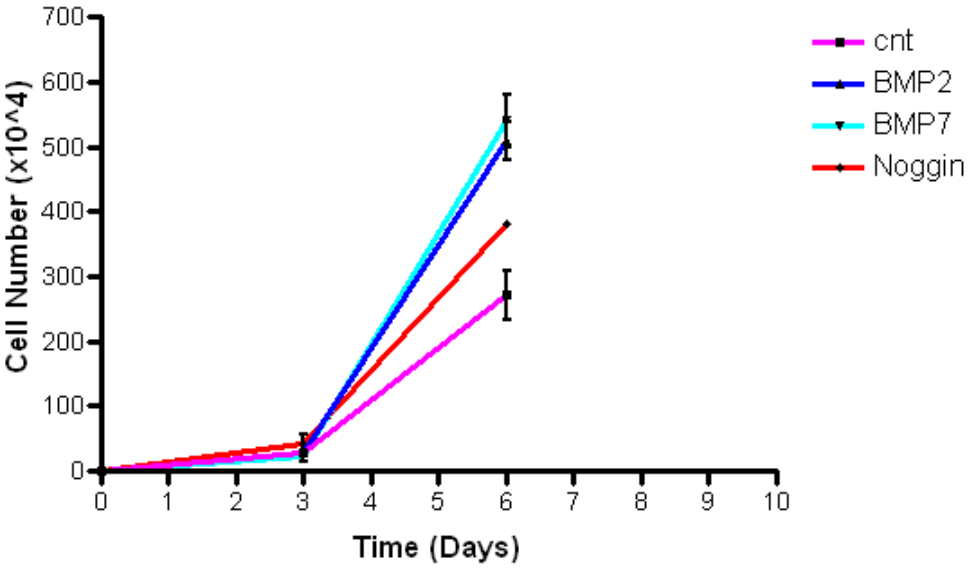



**Figure 5.9**. Cell Count of SW480 cells after 6 days of BMP2, BMP7, or Noggin treatment.

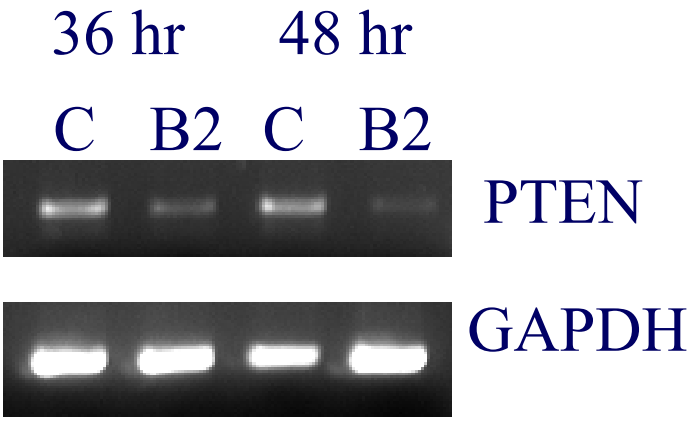



**Figure 5.10**. Semi-quantitative RT-PCR of PTEN mRNA after 36 and 48 hours of BMP2 treatment. GAPDH was used as a loading control.

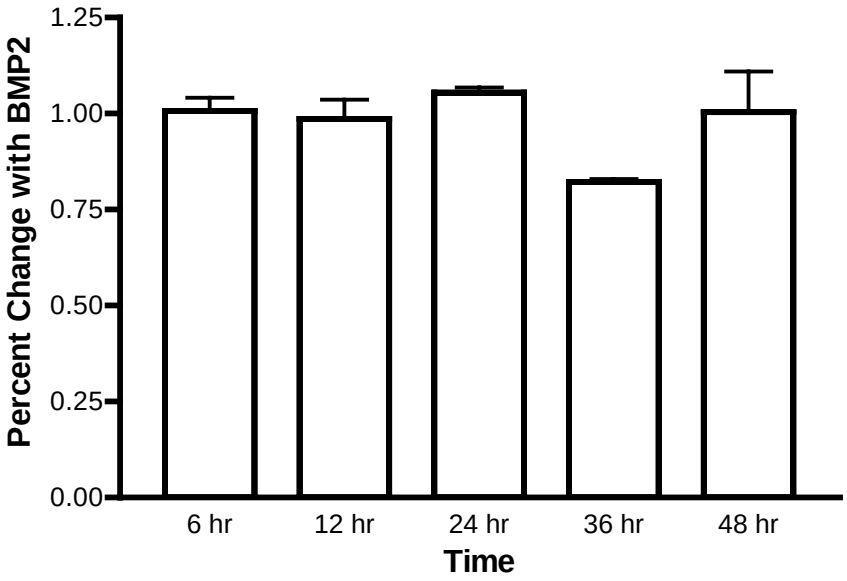



**Figure 5.11**. Luciferase assay time course using PTEN-luc. Expressed as Percent Change with BMP2 treatment from control.

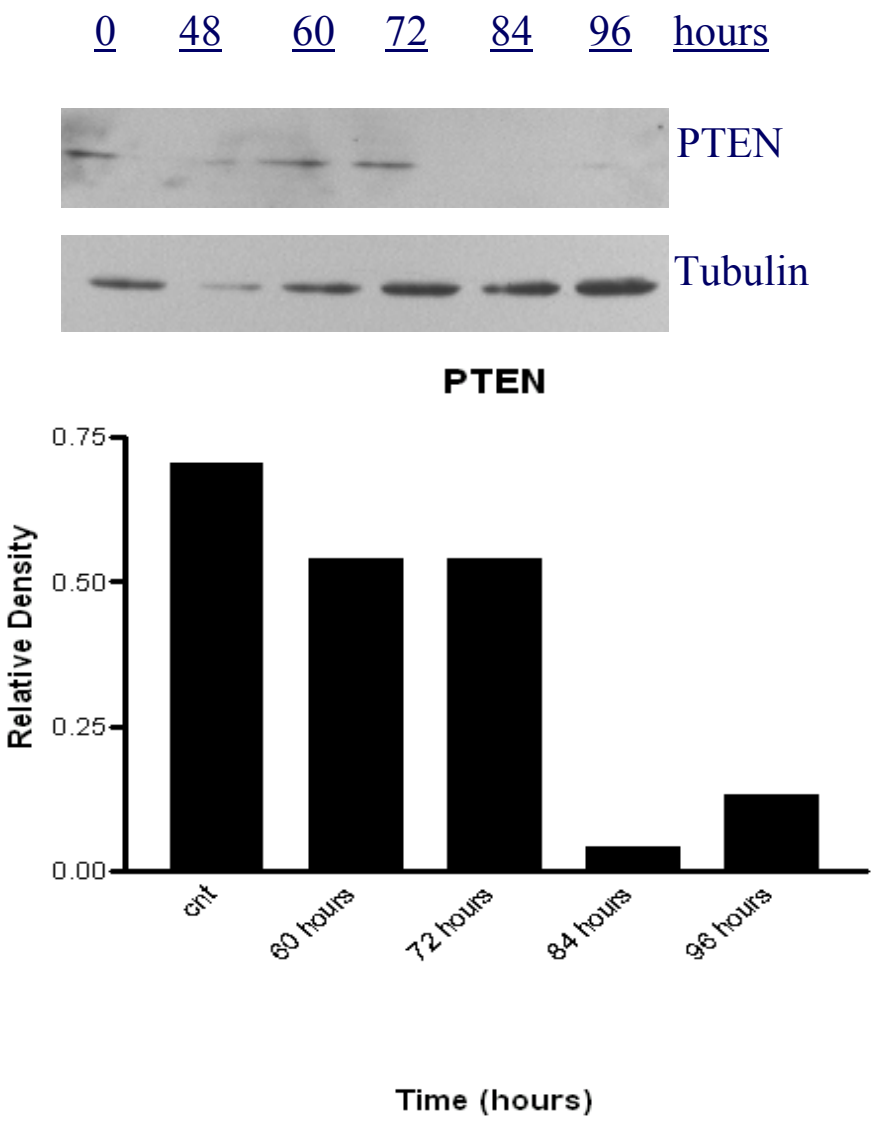



**Figure 5.12**. Western Blot of SW480 cells treated with BMP2 and probed for PTEN. Below is a densitometry graph representing the levels of PTEN protein present.

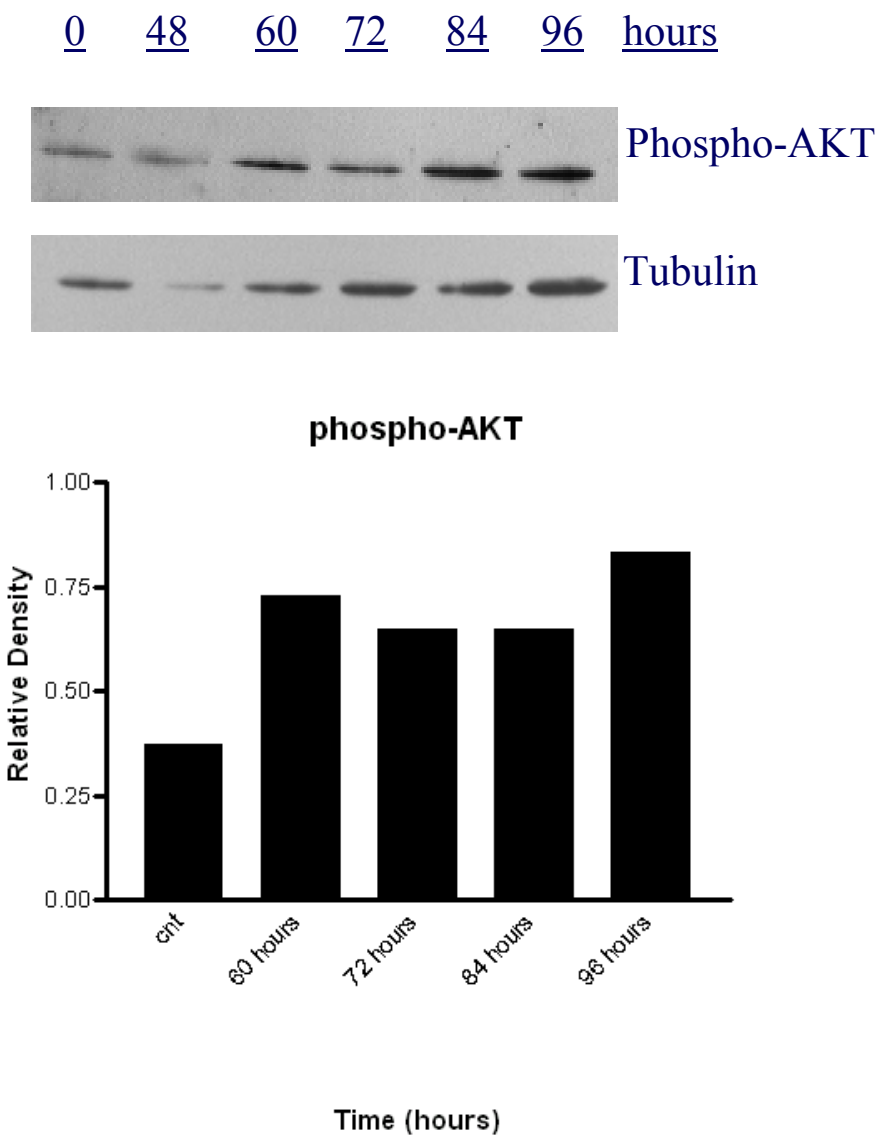



**Figure 5.13**. Western Blot of SW480 cells treated with BMP2 and probed for phospho-AKT. Below is a densitometry graph representing the levels of phospho-AKT protein present.

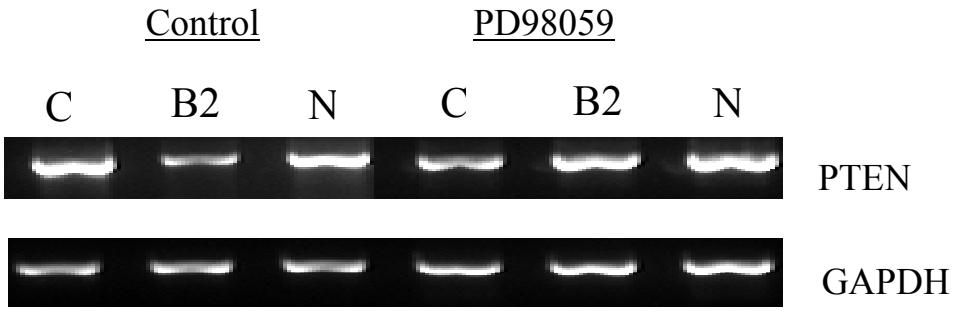



**Figure 5.14**. Semi-quantitative RT-PCR of SW480 cells treated with BMP2, noggin or PD8059 and BMP2 or Noggin. GAPDH is used as a loading control.

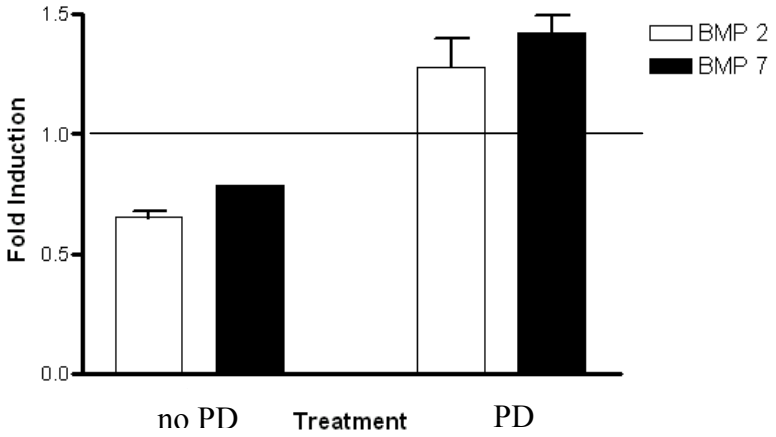



**Figure 5.15.** PTEN-luc assay on SW480 cells treated with or without 50 μM PD98059 for 36 hours, with or without 50 ng/ml of BMP2 or BMP7, as compared to untreated controls.

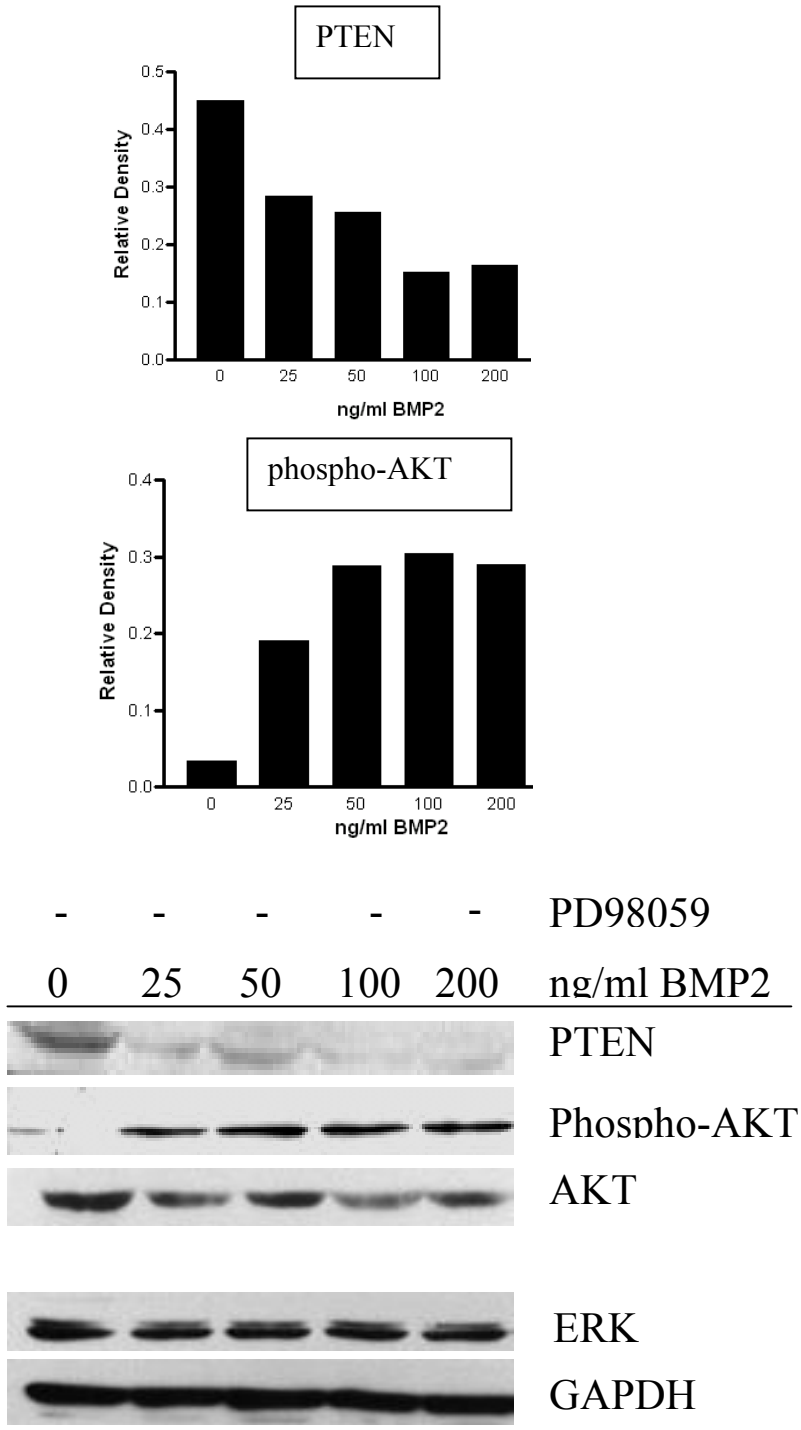



**Figure 5.16.** (a) Densometry of PTEN, (b) densometry of phospho-AKT relative to GAPDH, and (c) western blot of SW480 cells treated with increasing amounts of BMP2 for 84 hours.

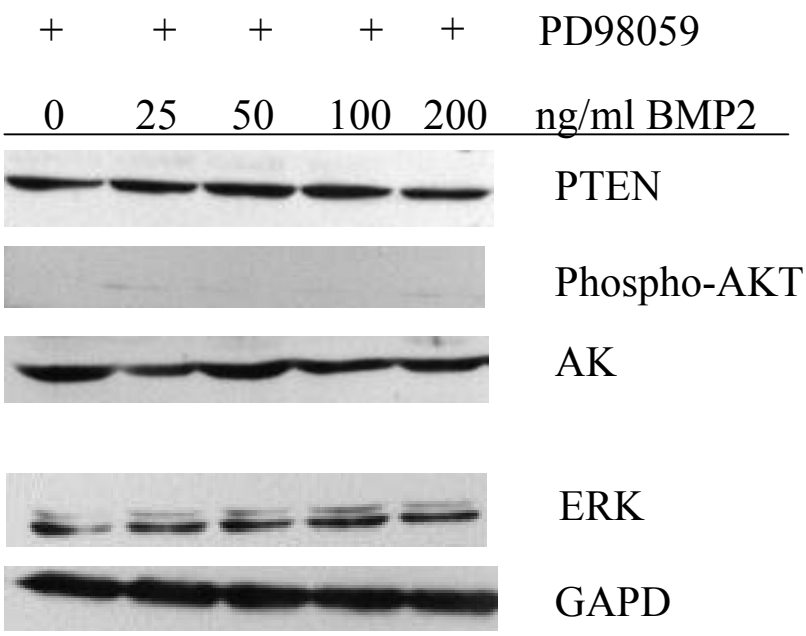



**Figure 5.17**. Western blot of SW480 cells treated with increasing amounts of BMP2 for 84 hours and 50 μM PD98059

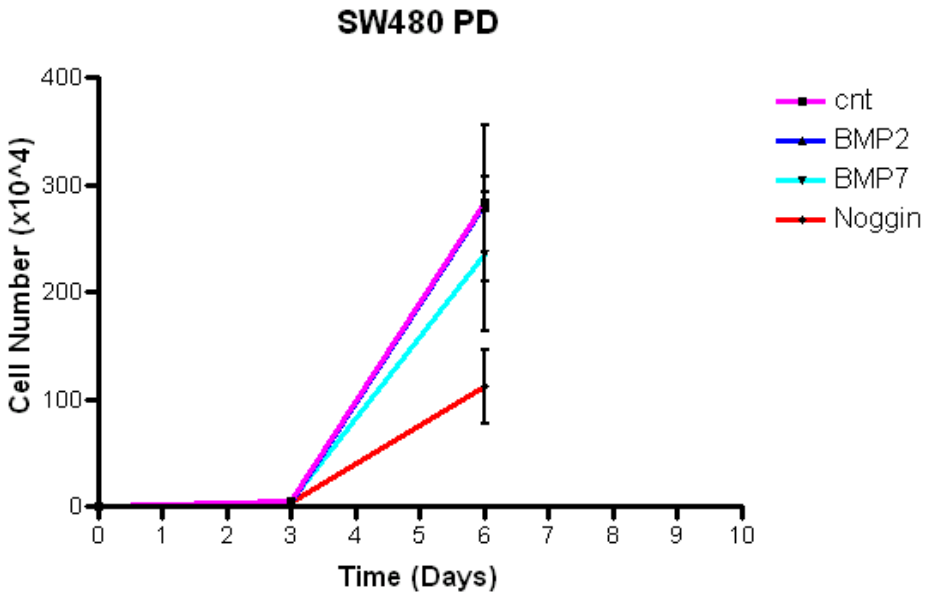



**Figure 5.18**. Cell Count of SW480 cells after 6 days of BMP2, BMP7, or Noggin treatment with PD98059.

# Chapter 6:

# Discussion

## Summary

The overall goal of the work in this dissertation was to determine what role, if any, BMP has in colon cancer. The BMP receptor IA is mutated in a colon cancer predisposition syndrome, Juvenile Polyposis. We found that the BMP2 and BMP7 ligands as well as the BMP receptor were intact and functional in human colon cancer tissues. We also found that the BMP SMAD signaling pathway was intact and growth suppressive to varying degrees depending on the genetic background of the colon cancer cell lines we examined. We determined that the BMP pathway interacts with the RAS pathway, and that active RAS prevents phospho-SMAD1 from entering the nucleus, reducing the functional effect of BMP ligand. Additionally, we found in a SMAD4-null colon cancer cell line that BMP switches from being growth suppressive to growth proliferative over time, in part by downregulating PTEN at the RNA and protein level. We found that this downregulation was modulated by the Erk Kinase pathway. Through the work done in this dissertation, we have begun to understand that the BMP pathway does in fact play an important role in colon cancer growth.

## BMP Signaling through the SMAD Pathway

BMPs are known to play a role in tissue development, but until recently, little work has been done examining the significance of BMP signaling in cancer. Work by several groups has previously shown BMP signaling to affect epithelial cell growth (Brubaker, 2004; Hardwick, 2004; Pouliot, 2002). TGFβ and activin ligands from the same superfamily as BMP, are known growth suppressors and have shown

to be inactivated in subsets of colon cancers (Jung, 2004; Grady, 1999; Markowitz, 1996; Hempen, 2003). BMP signaling can be inactivated by germline mutation of *BMPRIA* in the colon cancer predisposition syndrome, Juvenile Polyposis (Zhou, 2001; Howe, 2001). Taken together, these facts led us to hypothesize that the BMP pathway might be affected in colon cancer cell lines and human colon cancer specimens without Juvenile Polyposis. In this study, we investigated several colon cancer cell lines for the presence of intact BMP2 and BMP7 signaling and whether growth was affected by ligand treatment.

We determined that the BMPRIA receptor was present in all of the colon cancer cell lines and tissues examined. Thus, BMPRIA is expressed in colon cancers as opposed to some polyps from JP patients. To further evaluate whether the BMPRIA receptor was active, we found SMAD1 was phosphorylated and was transported to the nucleus in the colon cancer tissues and HCT116 cell line and its derivatives. Further, we utilized a luciferase reporter vector specific for BMP-induced SMAD signaling to confirm that BMP signaling could induce transcription. While we found several fold increases in transcriptional activity induced with ligand treatment in both the HCT116 (*TGFBR2* and *ACVR2* mutated) and HCT116+chr3 (*TGFBR2* reconstituted), the HCT116+chr2 cell line, which contains an extra copy of *BMPRII* and a functional copy of *ACVR2* had a higher transcriptional activity with ligand treatment over controls (Jung et al., 2004). We propose that the higher levels of BMP-induced transcription in HCT116+chr2 cells is a direct result of either one or both of the reconstituted receptors being present, with the ability to transduce the effects of BMP. Using DN BMPR1A transfection, we show inhibition of BMP-

induced SMAD dependent transcription, further indicating that this receptor is necessary for transducing the effects of the BMP ligands. The reduction in transcriptional activity as a result of DN BMPR1A transfection appeared to be greater in the HCT116+chr2 cells compared to the HCT116+chr3 cells, again likely due to the presence of additional receptors encoded on chromosome 2 that are involved in BMP signaling. Additionally, we found that upon stimulation with BMP2 and BMP7, HCT116, HCT116+chr2, and HCT116+chr3 exhibited modest but significant growth suppression as assayed by the MTT assay. The growth suppression was verified in our models with the use of DN BMPR1A to impair signaling through this receptor. Indeed, DN BMPR1A reversed the BMP-induced growth suppression we observed. These results are consistent with an intact and functional BMP pathway in these colon cancer cells, with the manifestation of growth suppression.

We also investigated whether BMP2 or BMP7 stimulation induced changes in cell viability or cell cycle progression. We did not find BMP2 and BMP7 stimulation to induce changes in cell viability in our system in contrast to what has been previously reported by other groups (Hardwick et al., 2004). Our cell cycle analysis did not identify any changes in cell cycle progression and our analysis of $p21^{WAF1}$ transcriptional activity did not demonstrate differences with BMP stimulation. Thus, the moderate growth suppression seen in these cells does not appear to be directly related to activation of $p21^{WAF1}$ consistent with results reported for breast cancer cell lines (Pouliot & Labrie, 2002).

While it was not a main focus of this study, we found no difference in BMP signaling between MSI-H and MSS tissues, as was expected since the BMPR1A receptor does not contain a coding microsatellite. This is in contrast to other TGF-β superfamily members, TFGβ and activin, which have their signaling inactivated by mutation within coding microsatellites in key surface receptors (Grady et al., 1999; Jung et al., 2004). Thus, we have no evidence that BMP would have differential effects on MSI-H tumors as compared to the TGFβ and activin pathways. BMP signaling could represent a potential pharmacological target that may lead to moderate growth suppression in both MSI-H and MSS tumors.

In conclusion, unlike other TGFβ-superfamily members such as TFGβ and activin signaling that are often inactivated in subsets of colon cancer, BMP signaling appears to be intact in these tumors. To our knowledge this is the first examination of BMP7-induced signaling in sporadic colon cancers. In juvenile polyps from patients with JP, loss of BMP signaling might contribute to the high risk of colon cancer observed over the lifetime of these patients. Given that BMP signaling appears to be growth suppressive, there are a few possibilities why BMP signaling remains intact in colon cancers. First, the degree of tumor suppression may be moderate and thus overcome by other genetic events that occur in colon cancers. Secondly, other signaling pathways may adversely affect BMP growth suppressive signaling, such that traditional SMAD signaling is abrogated without mutation of the components (consistent with our data on RAS/ERK). These findings indicate further exploration is necessary of the pathways, and potential cross-talk between the pathways should be investigated.

**RAS Inhibition of BMP Signaling**

RAS protein is activated in greater than 50% of colon cancers and appears to interfere with BMP2 signaling. We utilized the non-tumorigenic, but kRAS mutated FET colon cells, and demonstrated that (a) BMP signaling and transactivation is intact, (b) BMP2 induces growth suppression, (c) BMP2 stabilizes p21 protein, with minimal increases in p21 transcription, and (d) the growth suppressive functions of BMP2 are slowed by activated RAS/ERK, attenuating BMP2's effects. Thus, RAS/ERK is a negative regulator of BMP signaling, and appears to facilitate BMP2 induced p21 degradation to avert growth suppression.

It has been reported that the RAS pathway can prevent SMAD1 from entering the nucleus by causing its phosphorylation at the linker region in COS1 cells (Kretzschmar et al., 1997a). We utilized a luciferase assay for SMAD specific BMP signaling as well as phospho-SMAd1 immunofluorescence to show more phospho-SMAD1 enters the nucleus when the RAS pathway is inhibited. We also used the MTT assay and cell counting to show that BMP2 is growth suppressive in FET cells. Inhibition of the RAS pathway further enhanced the BMP2 induced growth suppression.

We examined whether PTEN or p21, both known growth suppressive proteins, are partially responsible for BMP2 induced growth suppression in FET cells. BMP2 treatment has been shown to upregulate p21 in breast, prostate, and gastric cancers (Brubaker et al., 2004; Ghosh-Choudhury et al., 2000a; Ghosh-Choudhury et al., 2000b; Pouliot & Labrie, 2002; Wen et al., 2004; Yue et al., 1999) and the mechanism has been presumed increased transcription of p21. In the

HCT116 family of colon cancer cell lines, with more modest BMP2-induced growth suppression, p21 levels were not elevated (Beck 2006). In FET cells with an activated RAS pathway, p21 transcription was also not induced, however we found p21 protein levels increased in response to BMP2. We demonstrated that BMP2-induces stabilization of existing p21 protein, limiting its degradation, allowing p21 to extend its function for growth suppression. Differences in the p21 response to BMP2 could be due to differing genetic backgrounds in the colon cancer cell lines.

Waite et al. found PTEN becomes stabilized with BMP2 treatment by decreasing its association with proteins involved in the degradative pathway, UbCH7 and UbC9 in MCF7 breast cancer cells (Waite & Eng, 2003a). We did not find any change in PTEN at the RNA or protein level after BMP2 treatment in FET colon cancer cells, suggesting that p21 is more important as a mediator of growth retardation than PTEN in these cells.

The RAS/ERK pathway was inhibited at many levels with chemical inhibitors to MEK1 , as well as a dominant negative k-RAS. We found that with RAS inhibition, we saw an increase in growth suppression and more phospho-SMAD1 signaling to the nucleus with BMP2 treatment. Additionally, we saw that when the RAS pathway was inhibited, p21 was further stabilized with BMP2 treatment. Therefore, we show that the RAS signaling pathway reduces BMP2's effects in FET colon cancer cells. Both activating mutation is RAS (Grady & Markowitz, 2002) and the RAS's apparent ability to dull or inhibit BMP2's effect on FET colon cancer cells by inhibiting or limiting the amounts of phospho-SMAD1

translocating to the nucleus and preventing p21 degradation allow for further growth stimulation by the RAS family.

RAS/ERK activation is normally regulated by ligand stimulation, turning on a mitogenic pathway that causes cellular proliferation (Grady & Markowitz, 2002). It has also been reported that BMP2 can stimulate the RAS pathway. Indeed, BMP2 activates ERK, counterintuitive to its SMAD-mediated and p21 mechanistic growth suppression. BMP2 treatment activated ERK, by 10 minutes after BMP2 treatment that persisted through 120 minutes. The phospho-ERK activation is in addition to the constitutive levels stimulated by oncogenic RAS in these cells. When the RAS/ERK pathway was inhibited by PD98059 we did not see an increase in phospho-ERK with BMP2 treatment, as was expected. We hypothesize that this ERK activation is a brake to regulate or limit BMP-SMAD signaling and growth suppression. On the other hand, oncogenic RAS, causing constitutive stimulation of this mitogenic pathway, continuously interferes with intact BMP-SMAD signaling, more permanently muting BMP-SMAD induced signaling and growth suppression.

In conclusion, we demonstrate that RAS/ERK can be activated by BMP2 signaling and that RAS/ERK attenuates BMP2-SMAD signaling and growth suppression in FET cells. The growth suppression is mediated through BMP-induced increases in p21 protein through enhanced stability of p21 and not through increased p21 transcriptional activity. The BMP-induced p21 stability is also reduced by activated RAS/ERK. We propose RAS/ERK activation, common in colorectal adenomas and cancer, as one mechanism to prohibit the growth suppressive effects of intact BMP signaling.

**SMAD4-independent BMP Signaling**

SMAD4 mutations occur in approximately 10% of in colon adenomas and nonmetastatic carcinomas (Riggins et al., 1997) but in 30% of invasive metastatic carcinomas and in colon cancer metastases . SW480 is a *SMAD4*-null colon cancer cell line, and upon reconstitution of *SMAD4*, there was strong transcriptional activation of BMP-regulated SMAD signaling in the absence of exogenous BMP ligands, indicating high autocrine stimulation of the BMP receptors. This was verified by treating *SMAD4*-transfected cells with noggin (which binds free ligand) and demonstrating a subsequent decrease in transcriptional activity, as well as the presence of BMP2 and BMP7 ligand transcripts that can make protein available for autocrine or paracrine activity upon the BMP receptors.

SW480 cells were also analyzed for their ability to close a wound scratched through the center of confluent cells, as well as the cells ability to invade through an EC matrix. In particular, BMP2 ligand treatment inhibited the ability of SW480 cells to close the wound and invade through the EC matrix. SW480 cells treated with Noggin were able to close the wound and to invade through the EC matrix to near the same extent as untreated SW480 cells and. Thus, BMP2 may slow growth and/or inhibit the invasive ability of BMP-sensitive colon cancer cells at early time points.

Furthermore, non-transfected SW480 cells exhibited significant decreases in growth at early time points when treated with BMP2 and BMP7, and BMP2-induced growth suppression was reversed with transfection with the DN BMPR1A vector. Interestingly, over time, BMP2 treatment switched from being growth suppressive to

growth proliferative. Taken together, these findings indicate that BMP can induce SMAD4-independent growth effects in SW480 cells by a yet, uncharacterized pathway. Levels of the tumor suppressor PTEN decreased with long-term BMP2 treatment (36 hours for the mRNA and 84 hours for the protein), which seems to corresponds with the switch from growth suppression to growth proliferation and might provide a possible mechanism for the observed growth switch in by BMP2. Additionally, we saw an increase in phospho-AKT levels with the decrease in PTEN levels, suggesting activation of the PI3K with downstream effectors induced by the BMP-induced decreases in PTEN levels.

Inhibition of the RAS/ERK pathway with PD98059 reversed the BMP2-induced decreases in PTEN levels and increases in phospho-AKT. Additionally, we no longer saw a change to growth proliferation with BMP2 treatment when the RAS/ERK pathway was inhibited with PD98059. These results suggest that BMP2 is modulated by the RAS/ERK pathway in a SMAD4-independent manner to decrease PTEN levels, increasing phospho-AKT levels, resulting in a switch from growth suppression to growth proliferation in the SW480 cells.

**Future Work**

While there has been a significant increase in the amount of knowledge known about BMP signaling in epithelial cells in the last ten years, there is still quite a bit to be discovered. One question plaguing the BMP field is the different roles of the various signaling BMPs. There are three main signaling BMPs, BMP2, BMP4, and BMP7, and most work previous work makes the assumption that these three

BMPs have the same effect in cells. It is likely that they have different roles depending on the context of the cell and are activated in response to different input signals. Additionally, another interesting area of research is the roles of the different BMP receptors. BMP ligand is able to signal through six different receptors and likely each of these combinations dictates the signal to the cell. The potential problems with these areas of research are that one would have to knock down all of the other receptors to be able to fully investigate the roles of each individual receptor. Additionally, one would have to knock down the other BMP ligands to examine the effects in the cell of the different ligands. Current technology makes it relatively hard to knock down more than two targets at a time, but likely in the near future this will be possible.

A further potential line of future investigation is the role of the different BMP receptor SMADs (Michiko Miyaki, 1999). Currently, there are three known BMP Receptor SMADs, SMAD1, SMAD5, and SMAD8. Most previous work in the field has been done assuming that these three SMADs have the same function. While it is likely that they have similar functions, it doesn't make evolutionary sense to have three proteins that have the exact same function and get activated from the same signal. It is more likely that these different SMADs get activated at different times based on the different signals to the cells. One possibility is that based on which BMP ligand stimulates the cell (BMP2, BMP4, or BMP7) a different SMAD gets activated, Other groups have shown that there are different physiological responses to BMP ligand depending on the extracellular concentration of ligand, and perhaps an explanation for this phenomenon is that different SMADs get activated in

response to extracellular concentrations of ligand, invoking slightly different extracellular responses. Our group and others have shown that other signaling pathways interact with the BMP pathway influencing the outcome of the BMP ligand on physiological function and perhaps the cumulative effect of other pathways also dictates which receptor SMAD gets activated.

Another line of future investigation involves further understanding the cross-talk and interactions of other signaling pathways with the BMP pathway. We have begun to show along with other groups that both the PI3 Kinase and RAS pathways interact with the BMP pathway. These interactions need to be further characterized, as well as interactions with other signaling pathways since all of these pathways are potentially communicating within the cell and it is the cumulative effect and balance of the input signals to the cell that determines how cells respond. One potential problem with this line of investigation is that technological shortcomings make it hard to currently examine individual pathways in mammalian cell models as well as to be able to attribute the function of a cell to the various signals dictating it in the complex signaling network occurring in a mammalian cell at any one time.

Perhaps one of the biggest questions raised by the work done in this dissertation left to be fully understood is how BMP initially inhibits growth in the SMAD4-null colon cancer cells. We found that BMP treatment was growth suppressive in SMAD4-null SW480 cells, but over time becomes growth proliferative. We determined that the growth proliferation in part occurs by decreases in PTEN levels through the Erk kinase, but it is still unclear what causes the initial growth suppression. It is generally believed that while BMP interacts with other

signaling pathways, BMP induced growth suppression occurs through the SMAD pathways. In these cells, we still observe growth suppression in the absence of SMAD4. One possible explanation is that SMAD1 is able to activate transcription of growth suppressive genes in the absence of SMAD4, either alone or by utilizing an as of yet undiscovered protein, but there is no current direct evidence to prove this. Another possibility is that the BMP ligand is able to stimulate another undetermined growth suppressive and/or proliferative pathway. There are many possibilities for how BMP might initially be growth suppressive in SMAD4-null colon cancer cells, all of which will challenge the developing dogma of how BMP ligand works to transmit its signal to the nucleus.

**Model/Conclusions**

Through the work performed in this dissertation, we have found that the BMP pathway is intact and growth suppressive in many colon cancer cell lines studied, but is modulated by other growth pathways including the RAS/ERK pathway. We show that BMP2 increases the stability of p21, and the RAS/ERK pathway acts to dull the growth suppressiveness and decrease the stability of p21. Additionally, when SMAD4 is not intact, BMP utilizes activated RAS/ERK to decrease levels of PTEN, and switch from growth suppressive to growth proliferative **(Figure 6.1)**. When the BMP-SMAD pathway is not intact, as is the case in SMAD4-null SW480 cells, BMP is still active in the cells, which triggers nuclear phospho-SMAD1 and which is attenuated by the RAS/ERK pathway. When SMAD4 is not intact in the cells, BMP2 is able to decrease PTEN levels with a corresponding increase in phospho-AKT

levels, as a mechanism to switch from growth suppression to proliferation. We have shown that this decrease in PTEN levels occurs in part via modulation by RAS/ERK pathway. Other groups have shown that in hamartomatous polyp syndromes, PTEN, BMPRIA and SMAD4 can all be exclusively mutated. The results found in this dissertation suggest a possible mechanism for how these disruptions in these three genes might lead to aberrant growth. Additionally, our results suggest that while the BMP pathway is often intact in colon cancer cell lines, other pathways modulate the effects of this pathway.

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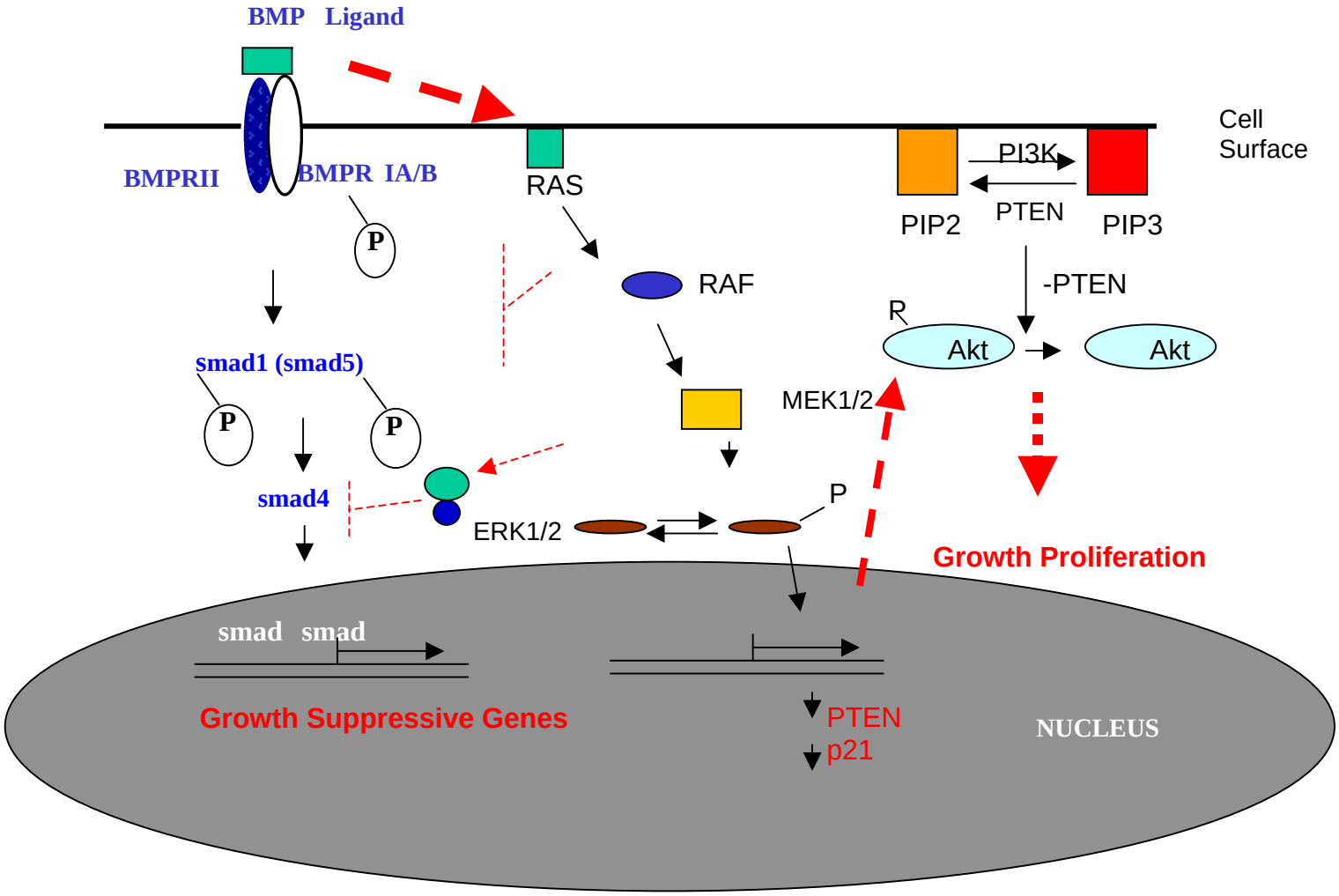


**Figure 6.1**. Summary model of BMP signaling in Colon Cancer Cell Lines.

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